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14. ABSTRACT

Based upon the results obtained from these studies, we can draw the following conclusions: 1) Airway hyperresponsiveness developed in Ova-sensitized mice was less pronounced in TRPV1-null mice, indicating an important role of TRPV1. 2) An increase in airway temperature within the normal physiological range triggered bronchoconstriction in sensitized rats, but not in control rats. Chronic airway inflammation in sensitized animals is likely a major contributing factor in causing this response. 3) A transient increase in airway resistance was generated immediately after hyperventilation with warm humid air in patients with mild asthma, but the same warm humid air challenge failed to cause any bronchoconstriction in healthy subjects. Furthermore, this bronchoconstriction is likely generated by the increase in airway temperature because hyperventilation with humidified air at room temperature did not generate any change in airway resistance in the same patients. These studies, once completed, should provide important and novel information for: 1) documenting the pulmonary stresses induced by hyperthermia in healthy individuals and in patients with sensitized airways; 2) understanding the mechanism underlying the hyperthermia-induced pulmonary dysfunction; and 3) detecting the susceptibility to heat stress in soldiers with underestimated or overlooked airway hypersensitivity such as in airway allergy or mild asthma.

15. SUBJECT TERMS
Hyperthermia, asthma, airway constriction, cough, dyspnea

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1. INTRODUCTION

Hypothesis: It is well documented that vagal bronchopulmonary C-fiber sensory nerves play an important role in the overall regulation of cardiopulmonary functions and protection of the airways against various environmental stresses. The primary hypothesis of this project is that the expression of the transient receptor potential vanilloid type 1 (TRPV1) channel is up-regulated in the airway mucosa of patients with mild asthma, allergic rhinitis and upper respiratory infection, which makes these patients more susceptible to the bronchoconstriction and other respiratory dysfunctions induced by thermal stress. Military Relevance: Soldiers in battle field are often subjected to severe and prolonged stress of hyperthermia. Sustained operations in hot and humid environment can debilitate physiological conditions of the soldiers, severely hinder both their physical and mental abilities to perform combat skills and operations, and lead to increased casualties.

2. Keywords

Hyperthermia, asthma, airway constriction, cough, dyspnea

3. Accomplishments

Objectives:

The specific aims of this proposal were to determine: 1) the acute effect of hyperthermia on airway function and the role of TRPV channels in triggering the bronchospasm; 2) whether this effect is heightened by acute airway inflammation; and 3) the temperature thresholds of thermal stress in generating airway dysfunction in healthy volunteers and in patients with mild asthmatics, allergic rhinitis and laryngopharyngeal reflux.

Major Accomplishments:

We have made major research accomplishments in this project. Based upon the results obtained from the studies in this project, we have reached the following important conclusions:

1) Hyperventilation of hot humid air triggered an immediate and reversible increase in airway resistance in patients with mild asthma, but caused either only a very small or no response in healthy subjects. The bronchoconstriction in these patients was completely prevented by pretreatment with ipratropium aerosol, indicating an involvement of cholinergic reflex. Accompanying the bronchoconstriction, breathing hot humid air also triggered coughs consistently in these patients, suggesting an involvement of the airway sensory nerves, presumably the thermosensitive TRPV1-expressing C-fiber afferents.

2) Hyperventilation of humid warm air triggered vigorous cough response and respiratory discomfort in patients with allergic rhinitis, indicating the involvement of the airway sensory

nerves. Chronic inflammation in the upper airways may have contributed to an up-regulation of the sensitivity and/or expression of TRPV1 in these sensory nerves.

3) Vigorous cough responses were triggered during hyperventilation with hot humid air in patients with laryngopharyngeal reflux, and these responses were absent in in healthy control subject. This effect of hyperthermia is probably mediated through an activation of the temperature-sensitive TRPV1 channel expressed on vagal bronchopulmonary C-fibers.

4) In an animal model of asthma (Brown-Norway rats sensitized by ovalbumin), chronic allergic inflammation sensitization increases the excitability of pulmonary C-fibers to chemical stimuli including both TRPV1 and non-TRPV1 activators. The hypersensitivity is further enhanced by an increase in the airway temperature.

5) An increase in tracheal temperature within the normal physiological range triggered a sustained but reversible increase in airway resistance in sensitized rats. In sharp contrast, this bronchoconstrictive effect of airway hyperthermia was not found in control rats.

6) The endogenous release of tachykinins from vagal bronchopulmonary C-fibers is responsible for the airway extravasation induced by increase in airway temperature, which is mainly mediated through activation of neurokinin-1 receptors.

These findings have provided strong evidence in support of our hypothesis that the stress of hyperthermia exerted on the respiratory system is primarily mediated through an activation of the temperature-sensitive TRPV1 channel expressed on vagal bronchopulmonary C-fibers, and that TRPV1 expression is up-regulated in the airway mucosa of patients with chronic inflammation. However, these important observations also clearly indicated that further studies will be required to uncover the underlying mechanisms and to develop the effective preventive and therapeutic approached in order to alleviate these respiratory dysfunctions.

Training and Professional Developments:

Not applicable.

Results Disseminated to Communities:

See Item #9 (Appendix).

Next Step to Accomplish Our Goals:

To continue the investigations that have been initiated in this project, new grant applications have been prepared and submitted to NIH, and the funding decisions will be made later this year.

4. Impact:

Our studies have made the following major impacts in the field of pulmonary research:

1) Established critical information for documenting the distinct difference in these airway responses to thermal stress between healthy individuals and patients with inflammatory airway diseases.

2) Demonstrated the involvement of TRPV1-sensoty nerves in eliciting these airway responses to thermal stress.

3) Obtained novel knowledge and developed new protocol for detecting the susceptibility to heat stress in individuals with underestimated or overlooked airway hypersensitivity, such as in patients with mild asthma, allergic rhinitis and laryngopharyngeal reflux.

5. Changes/Problems

No major problems or obstacles were encountered during the course of this project.

Two no-cost-extension requests were made for this project for the following reasons, which have led to some delays in our research progress: 1) Both Dr. Don Hayes and Dr. Deanne Roberts, the co-investigators of this project, left the University of Kentucky unexpectedly, and they had to be replaced. 2) A new group of patients with laryngopharyngeal reflux were proposed, approved and added to the study plan. Consequently, modifications of the IRB and HRPO of our study original protocols had to be approved before we initiated the proposed study.

6. Products

Eleven full papers have been published in peer-reviewed high-quality journals. A publication list is presented in **Item #9 (Appendix)** below.

7. Participants & Other Collaborating Organizations

Participants:

Lu-Yuan Lee, Ph.D., Principal Investigator (30% effort for all 3 years)
Don Hayes, M.D., Co-investigator (20% effort for year 1)
Mahdi Khosravi, M.D., Co-investigator (20% effort for years 2 & 3)
Paul B. Collins, B.S., RRT, Supervisor of Pulmonary Function Laboratory (5% effort for all 3 years)
Richard Kryscio, Ph.D., Consultant for Biostatistics (3% effort for all three years)
Ruei-Lung Lin, M.S., Research Analyst (100% effort for all three years)
Alice Hsu, Ph.D., Postdoctoral Fellow (100% effort for all three years)
Reyno Tapia, Undergraduate Part-time Lab Assistant
Robert Morton, Part-time Senior Research Analyst

Other Collaborating Organizations:

Not applicable.

8. Special Reporting Requirements

Collaborative Awards:

None.

Quad Charts:

See Item #9 (Appendix B)

9. Appendices

Appendix: The following publications were supported either in full or in part by this project. Their electronic links are included in this list below, and the PDF copies of these publications are submitted as attachments.

1. Hayes, D., P.B. Collins, M. Khosravi, R. L. Lin, and L.-Y.Lee. Bronchoconstriction triggered by breathing hot humid air in asthmatics: role of cholinergic reflex. Am. J. Resp. Crit Care Med. 185: 1190-6, 2012. http://dx.doi.org/10.1164/rccm.201201-0088OC
2. Lin, R.L., Y.J. Lin, M.J. Geer, R. Kryscio, and L.-Y. Lee. Pulmonary chemoreflex responses are potentiated by tumor necrosis factor alpha in mice. J. Appl. Physiol. 114:1536-43, 2013. http://dx.doi.org/10.1152/japplphysiol.01301.2012
3. Hsu CC, Lin RL, Lee LY, Lin YS. Hydrogen sulfide induces hypersensitivity of rat capsaicin-sensitive lung vagal neurons: role of TRPA1 receptors. Am J Physiol Regul Integr Comp Physiol. 305:R769-79, 2013. http://dx.doi.org/10.1152/ajpregu.00202.2013
4. Hsu CC, Lin RL, Lin YS, Lee LY. Bronchoconstriction induced by increasing airway temperature in ovalbumin-sensitized rats: role of tachykinins. J Appl Physiol (1985). 115:688-96, 2013. http://dx.doi.org/10.1152/japplphysiol.00491.2013
5. Lee LY, Gu Q, Xu F, Hong JL. Acid-sensing by airway afferent nerves. Pulm Pharmacol Ther. 26:491-7, 2013. http://dx.doi.org/10.1016/j.pupt.2013.03.010
6. Khosravi M, Collins PB, Lin RL, Hayes D, Jr., Smith JA, Lee LY. Breathing hot humid air induces airway irritation and cough in patients with allergic rhinitis. Respir Physiol Neurobiol. 198:13-9, 2014. http://dx.doi.org/10.1016/j.resp.2014.03.013
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9. Lin RL, Lin YJ, Xu F, Lee LY. Hemorrhagic hypotension-induced hypersensitivity of

vagal pulmonary C-fibers to chemical stimulation and lung inflation in anesthetized rats. Am J Physiol Regul Integr Comp Physiol. 308: R605-13, 2014. http://dx.doi.org/10.1152/ajpregu.00424.2014

10. Hsu, C.C., R.J. Tapia, and L.-Y. Lee. Airway extravasation induced by increasing airway temperature in ovalbumin-sensitized rats. Respir Physiol Neurobiol. 212-214: 46-49, 2015. http://dx.doi.org/10.1016/j.resp.2015.04.002
11. Lin YJ, Lin RL, Khosravi K, Lee LY. Hypersensitivity of vagal bronchopulmonary C-fibers induced by increasing airway temperature in ovalbumin-sensitized rats. Am J Physiol Regul Integr Comp Physiol. 309: R1285-91, 2015. http://dx.doi.org/10.1152/ajpregu.00298.2015

Appendix B: Quad Charts attached

Bronchoconstriction Triggered by Breathing Hot Humid Air in Patients with Asthma

Role of Cholinergic Reflex

Don Hayes, Jr.[1,2,3]*, Paul B. Collins[4], Mehdi Khosravi[2], Ruei-Lung Lin[5], and Lu-Yuan Lee[5]

[1]Department of Pediatrics, [2]Department of Internal Medicine, [3]Department of Surgery, [4]Pulmonary Function Laboratory, and [5]Department of Physiology, University of Kentucky Medical Center, Lexington, Kentucky

***Rationale*: Hyperventilation of hot humid air induces transient bronchoconstriction in patients with asthma; the underlying mechanism is not known. Recent studies showed that an increase in temperature activates vagal bronchopulmonary C-fiber sensory nerves, which upon activation can elicit reflex bronchoconstriction.**

***Objectives*: This study was designed to test the hypothesis that the bronchoconstriction induced by increasing airway temperature in patients with asthma is mediated through cholinergic reflex resulting from activation of these airway sensory nerves.**

***Methods*: Specific airway resistance (SR_{aw}) and pulmonary function were measured to determine the airway responses to isocapnic hyperventilation of humidified air at hot (49°C; HA) and room temperature (20–22°C; RA) for 4 minutes in six patients with mild asthma and six healthy subjects. A double-blind design was used to compare the effects between pretreatments with ipratropium bromide and placebo aerosols on the airway responses to HA challenge in these patients.**

***Measurements and Main Results*: SR_{aw} increased by 112% immediately after hyperventilation of HA and by only 38% after RA in patients with asthma. Breathing HA, but not RA, triggered coughs in these patients. In contrast, hyperventilation of HA did not cause cough and increased SR_{aw} by only 22% in healthy subjects; there was no difference between their SR_{aw} responses to HA and RA challenges. More importantly, pretreatment with ipratropium completely prevented the HA-induced bronchoconstriction in patients with asthma.**

***Conclusions*: Bronchoconstriction induced by increasing airway temperature in patients with asthma is mediated through the cholinergic reflex pathway. The concomitant increase in cough response further indicates an involvement of airway sensory nerves, presumably the thermosensitive C-fiber afferents.**

Keywords: asthma; cough; bronchoconstriction; TRPV1; ipratropium

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Recent studies suggest that increasing temperature within the physiological range can sensitize and stimulate C-fiber sensory nerves in the lung that express the thermosensitive transient receptor potential vanilloid type 1 channels (TRPV1). Activation of these sensory nerves is known to trigger various symptoms associated with airway inflammatory diseases, such as cough and bronchoconstriction.

What This Study Adds to the Field

This study suggests that hyperventilation of hot humid air evoked coughs and bronchoconstriction in patients with mild asthma but not in healthy subjects. The airway constriction is mediated through the cholinergic reflex pathway.

It is extensively documented that breathing cold dry air induces bronchoconstriction in patients with asthma, which results primarily from injury of airway mucosa and release of various bronchoactive autacoids, such as leukotrienes and histamine (1). In contrast, the effects of an increase in temperature on the airway functions in patients with asthma is generally overlooked despite the fact that hyperthermia occurs frequently under normal and pathophysiological conditions. The most common causes of hyperthermia are elevated metabolic rate (e.g., during exercise) and hindered heat dissipation (e.g., in a warm environment). Hyperthermia can also occur under disease conditions, such as in patients suffering from severe fever. Furthermore, tissue inflammation is known to lead to local hyperemia and an increase in tissue temperature in the inflamed area (2, 3). A recent study has reported that the average end-expiratory temperature plateau (as an indirect measurement of the lung tissue temperature) is 2.7°C higher in children with asthma than that in healthy control subjects (4).

An earlier study by Aitken and Marini (5) has shown that, after hyperventilation of the air with different combinations of temperature and humidity in patients with asthma, the most intense bronchoconstriction occurring immediately was generated by breathing hot humid air, which caused an almost 2-fold increase in airway constriction generated by cold dry air at the same time point. The bronchoconstriction caused by hyperventilation of cold dry air developed slowly and reached a peak after a delay of 5 to 10 minutes, whereas the bronchoconstrictive response to hot humid air developed much more rapidly in the same patients (5), suggesting a possible involvement of neural reflexes. However, this possibility was not tested in their study, and the underlying mechanism was not known.

A recent study in our lab has shown that vagal C-fiber sensory endings innervating the lungs were activated when the intrathoracic temperature was elevated to above a threshold of approximately 39.2°C (6). To avoid other indirect and complex effects of systemic hyperthermia, follow-up studies were performed in

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isolated vagal pulmonary sensory neurons; the results further demonstrated that a direct stimulatory effect of increasing temperature is mediated through activation of thermosensitive ion channels, namely the transient receptor potential vanilloid type (TRPV) receptors, expressed in these neurons (7–9). More importantly, stimulation of these TRPV-expressing bronchopulmonary C-fiber sensory nerves can elicit an array of pulmonary defense reflex responses, including cough and bronchoconstriction (10–12). Based upon the existing information, we hypothesized that hyperventilation of hot humid air increases airway temperature and evokes bronchoconstriction in patients with asthma by activating vagal bronchopulmonary C-fiber afferents. Furthermore, the airway constriction is mediated through cholinergic reflex pathways and therefore can be prevented by pretreatment with ipratropium bromide, a muscarinic receptor anatogonist, in these patients.

Some of the results of these studies have been previously reported in the form of an abstract (13).

METHODS

Subjects

Patients with asthma and healthy subjects were recruited by public advertisement. A screening test was performed in each subject after informed consent was obtained. The diagnosis of asthma was confirmed according to the standard clinical guidelines in each patient (14). Due to the need to stop therapeutic medications for 2 weeks before beginning the study, patients who had severe asthma or poor asthma control were excluded. The study protocol was approved by the Institutional Review Board at the University of Kentucky and the Human Research Protection Office of the United States Department of Defense.

Hyperventilation Challenge

A device designed to deliver air of desired temperature and humidity was constructed by the University of Kentucky Center for Manufacturing. Briefly, a humidified gas mixture of 4.5% CO_2 balance air at high temperature (HA; 49°C and 75–80% relative humidity measured by an Extech Hygro-Thermometer model RH101; Nashua, NH) or at room temperature (RA; 20–22°C and 65–75% relative humidity) was delivered at 300 L/min through a large-bore (3-in) stainless steel conduit. During the hyperventilation challenge, the subject breathed via a mouthpiece into this free stream of humidified gas mixture at approximately 40% of maximal voluntary ventilation for 4 minutes; CO_2 was added to maintain an isocapnic condition during hyperventilation. Humidity was generated from sterile isotonic saline by an ultrasonic atomizer (Sonaer Ultrasonics, Farmingdale, NY). The amounts of water content delivered in RA and HA were 12 to 14 and 56 to 60 mg H_2O/L of air, respectively. Levels of end-tidal temperature (time constant, 0.1 s) (Physitemp model IT-18; Clifton, NJ) and CO_2 (Novametrix 1260; Wallingford, CT) were measured before and after 2 minutes of hyperventilation when these changes reached steady state.

Pulmonary Function Measurements

Airway resistance (R_{aw}) and thoracic gas volume (V_{tg}) were measured by a whole-body constant-volume plethysmography (SensorMedics, Homestead, FL) for 6 minutes before and 16 minutes immediately after the hyperventilation challenge. During each measurement, the subject was asked to pant at a frequency of approximately 2 Hz for 8 s. R_{aw} and V_{tg} (with shutter closed) were determined by computer using the center-fit method for the slope measurement within the flow range of ±0.5 L/s for R_{aw}. Specific airway resistance (SR_{aw}) was calculated as: $R_{aw} \times V_{tg}$. Spirometry test was also performed along with the measurements of other physiological variables (body temperature, heart rate, arterial blood pressure, and oxygen saturation) before and after the challenge.

Study Design

Two study series were performed. In study 1, the responses to HA and RA hyperventilation challenges were compared in patients with asthma and in healthy subjects. In study 2, aerosolized ipratropium bromide (500 μg; 2.5 ml of 0.02% solution) and placebo (2.5 ml of sterile isotonic saline) were administered in a double-blind fashion, and their effects on the response to HA hyperventilation challenge were determined in patients with asthma.

Statistical Analysis

Unless noted otherwise, a two-way ANOVA was used for the statistical evaluation of the results. When the ANOVA showed a significant interaction, pairwise comparisons were made with a *post hoc* analysis (Tukey's test). Data are reported as means ± SEM. P values of < 0.05 were considered significant.

RESULTS

Six patients with asthma (21–26 yr of age; SD, 23 ± 1 yr) and six healthy subjects (19–46 yr of age; SD, 26 ± 4 yr) were enrolled in this study. Subject characteristics are shown in Table 1.

Study 1

The responses to HA and RA challenges were tested in a randomized order among subjects. Only one experiment was performed on a given day in each subject. In patients with asthma, hyperventilation of humidified HA did not change the end-tidal CO_2 concentration

TABLE 1. SUBJECT CHARACTERISTICS*

Patient Type, Subject No.	Age (*yr*)	Sex	Height (*cm*)	Weight (*kg*)	FEV_1 (*L*)	FEV_1 (*% of predicted normal*)†	FEV_1/FVC (%)
Asthma							
1	23	M	180	95	4.54	96	73
2	26	F	165	64	2.66	80	76
3	25	M	180	108	4.18	89	84
4	22	F	160	61	3.05	95	86
5	23	F	175	73	2.91	77	78
6	21	F	170	66	3.04	85	74
Healthy							
1	21	M	188	89	6.07	117	87
2	29	F	178	75	3.86	101	84
3	46	F	168	54	3.25	105	81
4	23	M	188	77	5.42	105	71
5	19	M	180	71	4.12	88	87
6	22	F	165	59	3.55	105	86

* Exclusion criteria for patients with asthma included chronic systemic corticosteroid use, exacerbation of asthma symptoms within the last month, other chronic lung diseases, acute respiratory illnesses within the last 6 wk, history of smoking, and congenital or acquired heart disease. Healthy subjects were nonsmokers who had no sign or previous record of pulmonary or cardiovascular disease.

† Predicted normal values obtained from Reference 40.

TABLE 2. CHANGES IN END-TIDAL TEMPERATURE AND CO_2 CONCENTRATION CAUSED BY HYPERVENTILATION OF HUMIDIFIED AIR AT ROOM AND HIGH TEMPERATURE*

	ET Temperature (°C)		ET CO_2 (%)	
Patient Type, Treatment	Before	During	Before	During
Asthma				
RA	33.5 ± 0.3	33.1 ± 0.3	4.49 ± 0.27	4.53 ± 0.34
HA	33.2 ± 0.2	34.7 ± 0.1†	4.52 ± 0.35	4.64 ± 0.28
Healthy				
RA	34.1 ± 0.6	33.0 ± 0.7	4.48 ± 0.36	5.11 ± 0.31
HA	32.7 ± 0.7	34.3 ± 0.6†	5.02 ± 0.24	4.82 ± 0.40

Definition of abbreviations: ET = end tidal; HA = humidified air at high temperature; RA = humidified air at room temperature.

* Measurements were made before and at 2 min after the beginning of the 4-min hyperventilation; the latter was measured immediately after the hyperventilation was interrupted for three to six breaths while the subject breathed room air during these measurements.

† Significant difference ($P < 0.05$; n = 6; paired *t* test) between before and during the hyperventilation challenge.

but generated a significant increase in end-tidal air temperature ($\Delta = 1.5 \pm 0.1$°C; $P < 0.05$) (Table 2). SR_{aw} increased immediately after hyperventilation and declined slowly toward baseline after more than 8 minutes (Figure 1A); the average SR_{aw} increased significantly from a baseline value of 7.09 ± 0.45 cm $H_2O \cdot s$ to a peak of 15.06 ± 2.29 cm $H_2O \cdot s$ after the HA challenge ($P < 0.05$) (Figure 2A). In the same patients, hyperventilation of humidified RA gas mixture did not cause a significant increase in SR_{aw} (baseline, 6.66 ± 1.49 cm $H_2O \cdot s$; peak response, 9.19 ± 1.41 cm $H_2O \cdot s$; $P > 0.05$) (Figures 1A and 2A). Wheezing in the chest was detected by auscultation during hyperventilation of HA in five of the six patients but was not detected in any patient during hyperventilation of RA.

In healthy subjects, hyperventilation of humid HA and RA gas mixtures caused similar changes in the end-tidal temperature and CO_2 concentration as those in patients with asthma (Table 2). However, in contrast to that in patients with asthma, the SR_{aw} responses were distinctly smaller, and there was no difference between the responses to HA and RA challenges in healthy subjects (Figures 1B and 2B). Wheezing was not detected during or after either of these challenges.

When the forced expiratory test was performed at approximately 8 minutes after the HA challenge, the ratio between FEV_1 and FVC was still significantly reduced ($P < 0.05$) from baseline (before HA challenge) in patients with asthma but not in healthy subjects (Table 3). The reduction was mainly generated by a reduced FEV_1 without a significant change in FVC. There was no significant change in residual volume after the HA challenge in patients with asthma (before: 1.34 ± 0.21 L; after: 1.53 ± 0.27 L; $P > 0.05$) or healthy individuals (before: 1.55 ± 0.20 L; after: 1.63 ± 0.32 L; $P > 0.05$).

In patients with asthma, the bronchoconstriction generated after the HA challenge was also clearly illustrated by a concave shape and a reduced peak flow in the expiratory flow-volume loops (e.g., Figure 3B), whereas RA challenge had no effect on the flow-volume loop in these patients (e.g., Figure 3A).

Hyperventilation of humidified HA also triggered coughs in five of the six patients with asthma, which began during the hyperventilation challenge and continued after the termination of HA challenge. Coughs were identified by the sound generated and verified by the short and sharp expiratory flow and pressure recorded at the mouthpiece in some of the experiments. The cough frequency increased from 0.11 ± 0.06 coughs per minute at the baseline to 2.19 ± 0.68 coughs per minute during the first 8 minutes after HA challenge (Figure 4A). In contrast, hyperventilation of RA rarely caused coughs in the same patients. In healthy individuals, neither HA nor RA hyperventilation generated cough (Figure 4B). The cough data during the hyperventilation challenge are not reported because they were not recorded in some of the subjects.

We used isotonic saline to humidify the inspired gas mixture in this study because inhalation of aerosolized distilled water is known to induce airway constriction in patients with asthma (15). In two of these six patients with asthma, we also tested their responses to HA challenge when humidity was generated by distilled water, but peak SR_{aw} and cough responses to HA hyperventilation were similar to those when humidity was generated by saline in these patients.

In patients with asthma, hyperventilation of HA caused small but significant increases in heart rate (baseline: 80 ± 4 beats/min; after HA challenge: 85 ± 5 beats/min; $P < 0.05$) and body temperature (baseline: 36.3 ± 0.2°C; after HA challenge: 36.6 ± 0.2°C; $P < 0.05$) but did not generate any significant

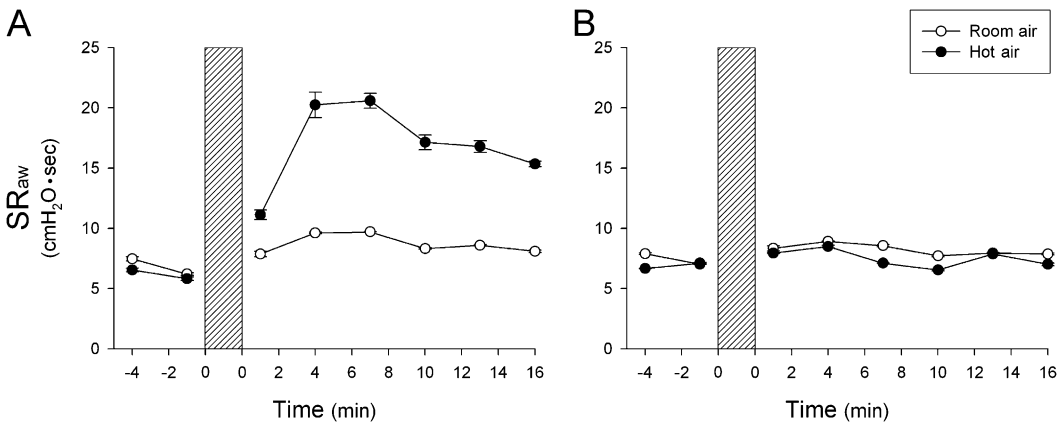

Figure 1. Representative responses of specific airway resistance (SR_{aw}) to hyperventilation of humidified room air (*open circles*) and hot air (*closed circles*) in a patient with asthma (*A*) and a healthy subject (*B*). Each point represents the data averaged over four consecutive breaths. During hyperventilation (*shaded bars*), the subjects breathed a gas mixture of 4.5% CO_2 balance air at 40% of maximal voluntary ventilation for 4 minutes. Only one experiment was performed in each subject on the same day. Data are means ± SEM of four breaths.

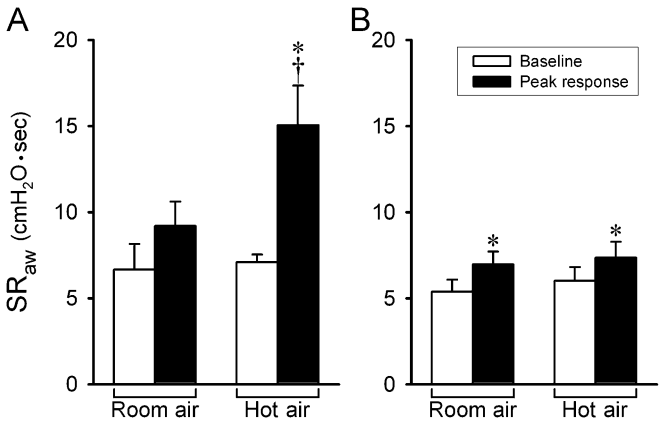

Figure 2. Group data showing a comparison of the peak responses of specific airway resistance (SR_{aw}) to hyperventilation of humidified room air and hot air in patients with asthma (n = 6) (*A*) and healthy subjects (n = 6) (*B*). Baseline and peak SR_{aw} were averaged over eight and four consecutive breaths before and after hyperventilation challenge, respectively, in each subject. Data are means ± SEM. *Significantly different ($P < 0.05$) from the baseline. †Significant difference ($P < 0.05$) comparing the corresponding data between room air and hot air.

change in blood pressure or oxygen saturation. Similar changes in heart rate and body temperature were found in healthy subjects after HA challenge.

Study 2

This study series was performed in the same patients with asthma tested in study 1. The HA challenge was studied 90 minutes after inhalation of ipratropium or placebo aerosol when ipratropium was expected to reach its full effect. Pretreatment with inhalation of ipratropium aerosol did not cause any change in heart rate ($P > 0.05$), blood pressure ($P > 0.05$), or any side effects (e.g., dizziness or blurred vision) in these patients but significantly reduced the baseline SR_{aw} (6.12 ± 0.72 cm $H_2O \cdot s$ before and 4.07 ± 0.58 cm $H_2O \cdot s$ after ipratropium; $P < 0.05$) (Figure 5A). More importantly, ipratropium prevented the increase in SR_{aw} generated by HA hyperventilation (peak SR_{aw} after HA challenge: 5.41 ± 0.62 cm $H_2O \cdot s$; $P > 0.05$) (Figures 5A and 5B). Pretreatment with placebo in the same manner did not change the baseline SR_{aw} (7.84 ± 1.59 cm $H_2O \cdot s$ before and 7.63 ± 1.58 cm $H_2O \cdot s$ after placebo; $P > 0.05$) and failed to prevent the HA-induced bronchoconstriction in the same patients (peak SR_{aw} after HA challenge: 13.90 ± 3.45 cm $H_2O \cdot s$; $P < 0.05$) (Figures 5A and 5B). The blocking effect of ipratropium pretreatment on the HA-evoked bronchoconstriction was also evident in the flow-volume loops obtained in these patients (e.g., Figures 3C and 3D).

Pretreatment with ipratropium, however, did not prevent the cough response elicited by the hyperventilation of humidified HA in patients with asthma (Figure 6). There was no difference in the cough frequency during the first 8 minutes after HA challenge between ipratropium and placebo pretreatments in the same patients (Figure 6).

The effect of ipratropium was not tested in healthy subjects because the HA hyperventilation challenge caused only a very small increase in SR_{aw} and because there was no difference between their responses to HA and RA challenges.

DISCUSSION

The results of this study showed that hyperventilation of HA triggered an immediate and reversible increase in airway resistance in patients with mild asthma but caused either only a very small or no response in healthy subjects. More importantly, the bronchoconstriction in these patients was completely prevented by pretreatment with ipratropium aerosol, indicating an involvement of cholinergic reflex. Breathing HA also triggered coughs consistently in these patients, suggesting an involvement of the airway sensory nerves that are responsible for eliciting the cough reflex. Furthermore, this reflex bronchoconstriction is likely generated by the increase in airway temperature because hyperventilation of RA did not generate any change in airway resistance in the same patients.

The mechanisms underlying these responses are not fully understood, but several factors possibly involved should be carefully considered. One of these factors is the activation of specific types of airway sensory nerves that are sensitive to an increase in temperature. The entire respiratory tract (from larynx to alveolar wall) is extensively innervated by vagal sensory nerves, of which the majority (~75%) are nonmyelinated (C-) afferent fibers (16). It is well documented that these bronchopulmonary C-fibers can be activated by various inhaled irritants and certain endogenous chemical mediators and play an important role in protecting the lung under normal and disease conditions (10–12). Stimulation of these afferents triggers bronchoconstriction, cough, mucus secretion, and other cardiopulmonary reflex responses in various species, including humans (10, 11, 17, 18). One of the most prominent characteristics of these bronchopulmonary C-fiber afferents is the expression of TRPV type 1 (TRPV1) receptors at the sensory nerve terminals (19, 20).

TRPV1, a member of the superfamily of TRP ion channel proteins, is a tetrameric membrane protein that forms a nonselective, non–voltage-gated cation channel (21, 22). TRPV1 and three other subtypes of TRPV channels (TRPV2–4) are known as the primary sensors for detecting warm temperature in mammalian species (23–25). Indeed, a recent study in our laboratory has

TABLE 3. CHANGES IN FORCED EXPIRATORY VOLUMES CAUSED BY HYPERVENTILATION OF HUMIDIFIED AIR AT HIGH TEMPERATURE AND AT ROOM TEMPERATURE

	FEV_1* (*L*)		FVC (*L*)		FEV_1/FVC	
Patient Type, Treatment	Before	During	Before	During	Before	During
Asthma						
RA	3.52 ± 0.29	3.44 ± 0.30	4.35 ± 0.46	4.43 ± 0.46	82.33 ± 3.24	78.67 ± 3.42*
HA	3.45 ± 0.32	3.04 ± 0.21*	4.30 ± 0.46	4.29 ± 0.41	81.17 ± 2.94	71.67 ± 2.36*
Healthy						
RA	4.27 ± 0.32	4.44 ± 0.49	5.39 ± 0.65	5.31 ± 0.65	81.20 ± 4.41	81.60 ± 4.72
HA	4.43 ± 0.48	4.48 ± 0.55	5.43 ± 0.72	5.43 ± 0.71	83.40 ± 4.37	83.40 ± 3.83

Definition of abbreviations: HA = humidified air at high temperature; RA = humidified air at room temperature.

*Forced expiratory tests were performed before and at ~8 min after the hyperventilation challenge in patients with asthma (n = 6) and healthy subjects (n = 5; the test was not done after HA in one subject).

†Significant difference ($P < 0.05$; paired *t* test) between before and after the hyperventilation challenge.

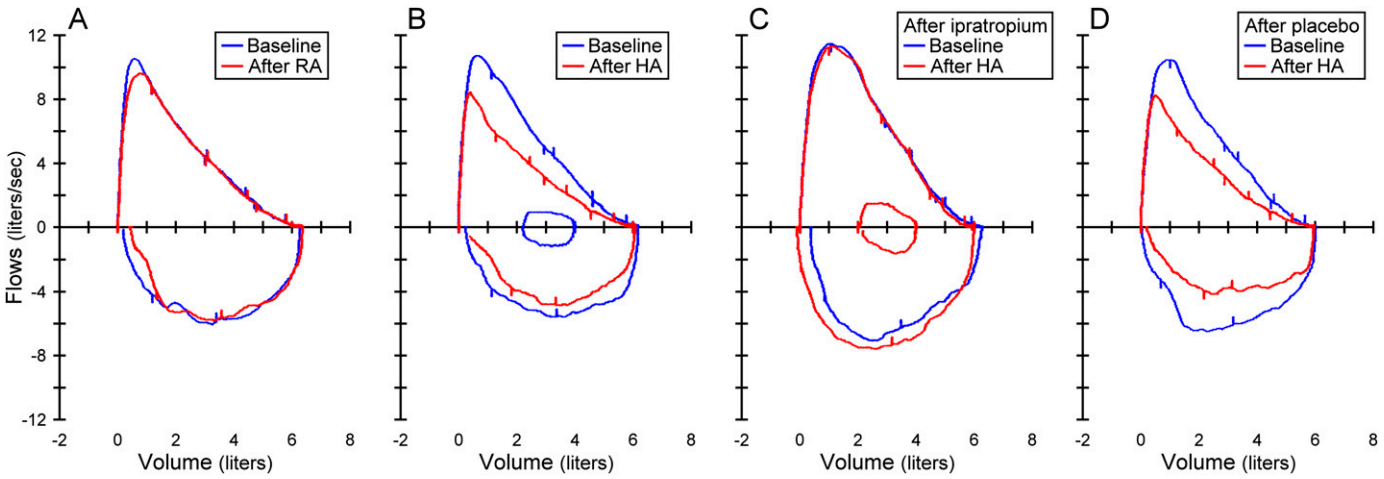

Figure 3. Effect of hyperventilation of humidified room air (RA) and hot air (HA) on forced expiratory flow-volume loops in a patient with asthma: baseline (*blue lines*) and response after hyperventilation challenge (*red lines*).

demonstrated that the afferent activity of these vagal pulmonary C-fiber endings was elevated when the temperature in the isolated perfused thoracic chamber was raised to above a threshold of approximately 39.2°C in anesthetized rats (6). More recent studies using the whole-cell perforated patch clamping technique have shown that an increase in temperature within the normal physiological range (35–41°C) evoked inward currents (voltage-clamp mode) and membrane depolarization and action potentials (current-clamp mode) in isolated pulmonary sensory neurons (7). These responses were reduced by more than 50% after pretreatment with AMG 9810, a selective antagonist of the TRPV1 channel, indicating that hyperthermia can exert a direct stimulatory effect on pulmonary sensory neurons and that this effect is mediated primarily through activation of the TRPV1 channel (7). More importantly, these bronchopulmonary sensory neurons were activated by increasing temperature to the level considerably lower than 43°C, which was initially reported as the temperature threshold for activating the heterologously expressed TRPV1 receptor (21).

A recent study reported that the average end-expiratory temperature plateau in children with asthma is 2.7°C higher than that in healthy individuals. Furthermore, the increased temperature is closely correlated with the number of eosinophils in induced sputum, indicating a coupling between the lung tissue temperature and the degree of inflammation in asthmatic airways (4). On the basis of results obtained in our previous studies described above (6, 7, 9), we suggest that the increase in airway tissue temperature due to inflammatory reaction can activate bronchopulmonary C-fiber sensory nerves during asthma exacerbation, which may play a part in the manifestation of various asthma symptoms, such as cough and bronchoconstriction. This possibility and the degree of its contribution remain to be tested.

In this study we used HA as a tool to raise the airway temperature, and an air temperature of 49°C was chosen to mimic that used by Aitken and Marini in their original study (5). Although this temperature is relatively high compared with the range of environmental temperatures, it is conceivable that the same elevation in airway tissue temperature as that in the present study can be generated by breathing HA at a lower temperature when the exposure time is lengthened. Surprisingly, it only generated a small increase of 1.5 to 1.6°C in the end-tidal temperature plateau in patients with asthma and in healthy subjects, probably due to the short duration (4 min) of exposure. We did not make direct measurement of the increase in local temperature in the airway tissue after HA hyperventilation, but we believe that it was probably underestimated by the measured increase in end-tidal temperature. Although we do not know if the increase in airway temperature induced by breathing HA in this study was sufficient to activate the TRPV1 in the airways, a recent report by Gavva and coworkers (26) has lent strong support to this possibility. Their clinical trial studies showed that treatment with the TRPV1 antagonist caused a significant increase in body temperature in healthy human volunteers, indicating that TRPV1 is active even at the normal resting body temperature and that the tonic activity of TRPV1 is involved in regulating normal core temperature (26). Hence, a slight increase in tissue temperature is expected to activate these channels. However, because the involvement of TRPV1 was not directly tested in this study, we cannot make a more definitive conclusion on its possible role in eliciting the responses observed in this study.

An important question remains as to why the same hot humid air challenge triggered cough and reflex bronchoconstriction in patients with asthma but not in healthy subjects. In addition,

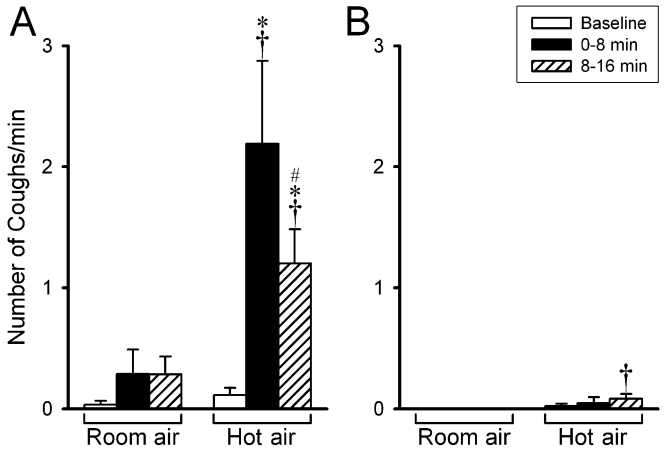

Figure 4. Group data showing a comparison of the cough responses to hyperventilation of humidified room air and hot air in patients with asthma (n = 6) (*A*) and healthy subjects (n = 6) (*B*). Cough frequencies were averaged in 8-minute durations before and after hyperventilation challenge in each subject. Data are means ± SEM and were collected in the same experiments as performed in Figure 2. *Significantly different ($P < 0.05$) from the baseline. †Significant difference ($P < 0.05$) comparing the corresponding data between room air and hot air. #Significantly different ($P < 0.05$) from that in the first 8 minutes after hyperventilation.

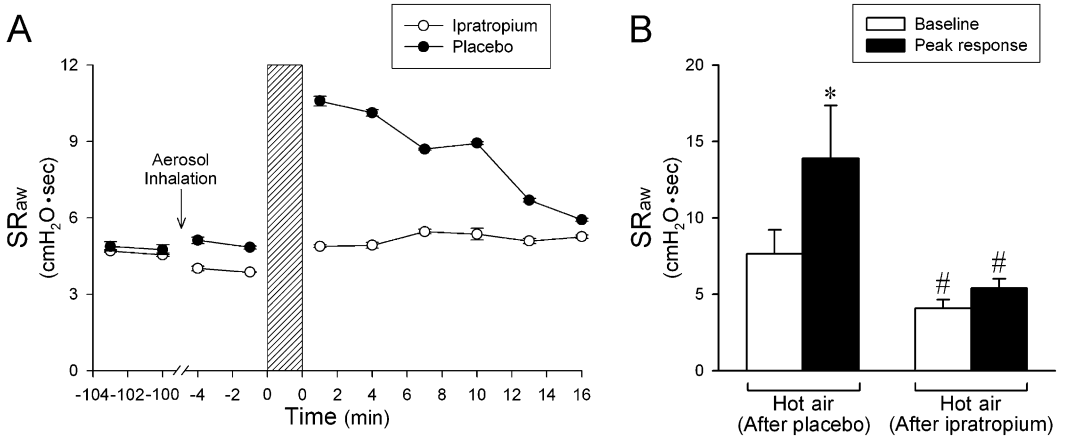

Figure 5. Effect of pretreatment with ipratropium aerosol on the airway response to hot humid air challenge in six patients with asthma. (*A*) Representative specific airway resistance (SR_{aw}) responses obtained in a patient with asthma. Ipratropium and placebo aerosols were administered in a double-blind fashion, and the response to hot air challenge was tested 90 minutes after the aerosol inhalation. The two tests were performed about 1 week apart. (*B*) Average data collected from all six patients with asthma comparing the peak SR_{aw} responses to hot humid air challenge after ipratropium and placebo pretreatments. Baseline and peak SR_{aw} were averaged over eight and four consecutive breaths before and after hyperventilation of hot air, respectively, in each subject. Data are means ± SEM. *Significantly different ($P < 0.05$) from the baseline. #Significant difference ($P < 0.05$) comparing the corresponding data between ipratropium and placebo pretreatments.

fever is not known to induce cough or bronchoconstriction in individuals without concurrent airway inflammatory diseases. Increasing evidence suggests an important role of bronchopulmonary C-fiber sensory nerves in the manifestation of various symptoms associated with airway inflammation, such as coughs and bronchoconstriction (27–29). Indeed, in addition to its role as a thermal sensor, TRPV1 can be activated by a number of endogenous chemical mediators, such as lipooxygenase metabolites and hydrogen ions, that are detected in the bronchoalveolar lavage fluid, sputum, or exhaled breath condensate of patients with inflammatory airway diseases (27, 30, 31). Moreover, cough sensitivity to the TRPV1 activators, capsaicin and citric acid aerosol, was markedly elevated in patients with asthma and chronic obstructive pulmonary disease (32). This is not surprising because certain endogenous inflammatory mediators (e.g., prostaglandin E_2, bradykinin) can markedly enhance the sensitivity of TRPV1 and lower its threshold for activation (28). Recent studies further revealed that increased expression of the TRPV1 in bronchopulmonary sensory nerves may be responsible, at least in part, for the increased TRPV1-mediated responses in allergic airway inflammation (33, 34). Whether there is an enhanced sensitivity or an increased expression of TRPV1 in the airways during chronic inflammation, the same stimulation, such as an increase in airway temperature, is expected to evoke a greater afferent discharge of the bronchopulmonary C-fiber sensory nerves and consequently more intense reflex responses (e.g., cough and bronchoconstriction). The bronchoconstrictive response may be further amplified in asthmatic airways due to the hypertrophy and hyperplasia of airway smooth muscles known to develop during the process of chronic inflammation–induced airway remodeling (35).

Cough is a protective reflex function elicited by chemical and/or physical activation of the cough receptors that are specific types of sensory endings innervating the respiratory tract. Our study showed that ipratropium completely prevented the bronchoconstriction (Figure 5) but did not prevent the cough response triggered by the HA inhalation challenge in patients with asthma (Figure 6), suggesting that the cough reflex was elicited by a direct activation of the cough receptors and not by an indirect effect generated by bronchoconstriction. Several important new findings of the physiological properties and morphological characteristics of these cough receptors have been described in recent reviews (36). It is generally recognized that the vagal bronchopulmonary C-fiber sensory ending is one type of these cough receptors. Indeed, aerosolized capsaicin, a selective activator of TRPV1, is one of the most effective tussive agents commonly used for testing cough sensitivity in humans (17). Our observation that increasing airway temperature consistently triggered coughs in patients with asthma but not in healthy individuals supports the hypothesis that the sensitivity and/or expression of TRPV1 are up-regulated in the airways of patients with asthma.

In their study, Aitken and Marini (5) clearly described the important role of water content contained in HA for delivering the "heat load" to the airways. Sheppard and coworkers have reported a bronchoconstriction triggered by inhalation of distilled water or hypoosmolar saline aerosols in patients with asthma (15, 37). This response was attenuated by atropine, indicating the involvement of the cholinergic reflex (15). Indeed, it was later discovered that distilled water can stimulate C-fiber afferents and

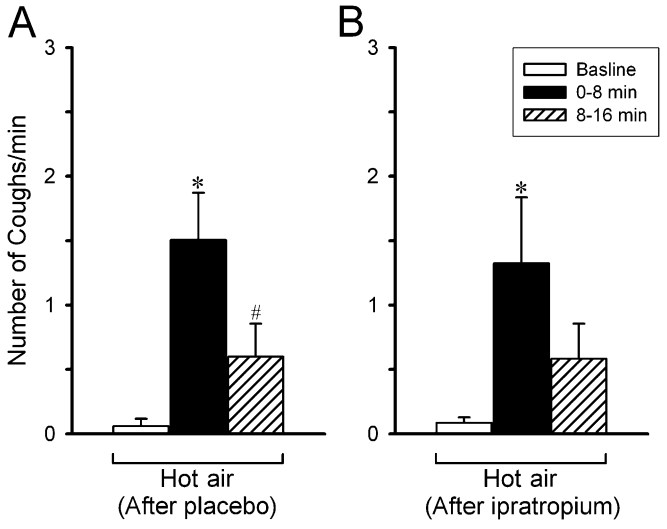

Figure 6. Group data showing a comparison of the cough responses to hot humid air hyperventilation challenge after placebo (*A*) and ipratropium (*B*) pretreatments in six patients with asthma. Cough frequencies were averaged in 8-minute durations before and after hyperventilation challenge in each subject. Data are means ± SEM (n = 6) and were collected in the same experiments performed in Figure 5. For further details, see legend of Figure 5. *Significantly different ($P < 0.05$) from the baseline. #Significantly different ($P < 0.05$) from that in the first 8 minutes after hyperventilation. There was no significant difference ($P > 0.05$) in the corresponding data between ipratropium and placebo pretreatments.

rapidly adapting receptors in the airways due to the low chloride ion concentration (38, 39). However, a possible involvement of the stimulatory effect of water can be ruled out in our study for the following reasons: (*1*) the humidity was generated from isotonic saline, which did not cause bronchoconstriction in the study by Sheppard and colleagues (15); (*2*) the amount of water content contained in the HA in our study was relatively low compared with that delivered by aerosol in their study (15); and (*3*) there was no detectable difference in the two patients with asthma tested in this study in their responses to hyperventilation of HA whether the humidity was generated from isotonic saline or distilled water.

In summary, this study showed that hyperventilation of HA triggered coughs and a reflex bronchoconstriction in patients with mild asthma but not in healthy individuals. The bronchoconstriction is mediated through the cholinergic reflex, suggesting that the airway sensory nerves, presumably the bronchopulmonary C-fibers, are activated by an increase in airway tissue temperature in these patients.

Author disclosures are available with the text of this article at www.atsjournals.org.

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Pulmonary chemoreflex responses are potentiated by tumor necrosis factor-alpha in mice

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Lin R-L, Lin Y-J, Geer MJ, Kryscio R, Lee L-Y. Pulmonary chemoreflex responses are potentiated by tumor necrosis factor-alpha in mice. *J Appl Physiol* 114: 1536–1543, 2013. First published March 28, 2013; doi:10.1152/japplphysiol.01301.2012.—Inhalation of tumor necrosis factor-alpha (TNF-α), a proinflammatory cytokine, induces airway hyperresponsiveness, and the underlying mechanism is not fully understood. Hypersensitivity of vagal bronchopulmonary C-fiber afferents is known to contribute to the airway hyperresponsiveness during an airway inflammatory reaction. Because activation of these afferents can elicit pulmonary chemoreflexes, this study was designed to determine if a pretreatment with TNF-α induced airway inflammation and enhanced the pulmonary chemoreflex sensitivity in anesthetized mice; and if so, whether the effect was mediated through activation of either or both of the TNF receptors, p55 and p75. Our results showed that TNF-α instilled into the lung caused an increased sensitivity of pulmonary chemoreflex responses to various chemical stimulants of the vagal bronchopulmonary C-fiber afferents. The increased sensitivity was found 24 h later, persisted at 48 h, and then gradually declined after several days. The TNF-α-induced airway hypersensitivity was accompanied by airway inflammation as shown by a striking elevation of the levels of eosinophils and neutrophils, several potent bronchoactive inflammatory mediators, and proinflammatory cytokines in the bronchoalveolar lavage fluid. Furthermore, the increase in pulmonary chemoreflex response caused by TNF-α was partially abrogated in both p55-null and p75-null mice, but completely abolished in p55/p75-null mice. In conclusion, TNF-α pretreatment induced airway inflammation and a sustained elevation of pulmonary chemoreflex sensitivity, which was mediated through an activation of both types of TNF receptors.

airway inflammation; airway reflex; C-fibers; transient receptor potential vanilloid type 1; asthma

AN IMPORTANT ROLE OF tumor necrosis factor-alpha (TNF-α), a proinflammatory cytokine, in the pathogenesis of allergic asthma has been widely recognized (4, 20, 42). TNF-α was detected in bronchoalveolar lavage fluid (BALF), sputum, exhaled breath condensate, and serum of asthmatic patients during asthmatic attack or following antigen inhalation challenge (25, 36). TNF-α is co-released with certain inflammatory mediators such as histamine and tryptase from a number of inflammatory cells in the airways (e.g., macrophages, mast cells, etc.) and can induce diverse and potent inflammatory responses by enhancing the release of various proinflammatory/chemotactic mediators and facilitating the infiltration of neutrophils and eosinophils into the airways (6, 28, 42). Indeed, inhalation of TNF-α can induce airway hyperresponsiveness accompanied by airway inflammation in healthy humans (43). A recent study has further demonstrated that prolonged (24–48 h) incubation of isolated rat pulmonary nodose and jugular sensory neurons with TNF-α induced a pronounced potentiating effect on the sensitivity of these sensory neurons to a number of chemical activators, particularly those activating the transient receptor potential vanilloid type 1 (TRPV1) receptor (21). However, the effect of TNF-α on the sensitivity of these TRPV1-expressing pulmonary sensory nerves in intact animals is not known.

TRPV1 is predominantly expressed in nonmyelinated (C-) sensory fibers. The C-fiber afferents innervating the respiratory tract play an important role in regulating cardiorespiratory functions under both normal and abnormal physiological conditions (12, 31). Furthermore, hypersensitivity of vagal bronchopulmonary C-fibers is known to contribute to the airway hyperresponsiveness during airway inflammatory reaction (24). Because activation of these C-fiber afferents can elicit pulmonary chemoreflexes in intact animals (12, 31), we hypothesized that, if a pretreatment with TNF-α enhances the sensitivity of these C-fiber afferents, it should augment the pulmonary chemoreflex responses elicited by chemical stimulants. Therefore, this study was designed to determine: *1*) if a pretreatment with TNF-α elevated the sensitivities of pulmonary chemoreflex responses to both TRPV1 and non-TRPV1 activators in anesthetized mice; *2*) if so, whether the increased sensitivity was associated with airway inflammatory reaction induced by the TNF-α pretreatment; and *3*) the role of TNF receptors (TNFRs) in the potentiating effects of TNF-α on pulmonary chemoreflex responses.

MATERIALS AND METHODS

The procedures described below were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health, and were also approved by the University of Kentucky Institutional Animal Care and Use Committee.

Animal preparations. The experiments were carried out in four groups of young adult mice (3–4 mo old) as follows: *1*) wild-type (WT; B6129SF2/J); *2*) TNF-α receptor double homozygous mutant mice (129S-*Tnfrsf1a*^{tm1Imx}*Tnfrsf1b*^{tm1Imx}/J) in which both types of TNFRs, TNFR1 (p55) and TNFR2 (p75), were mutated (p55/p75-null); *3*) p55-null mice (C57BL/6-*Tnfrsf1a*^{tm1Imx}/J); and *4*) p75-null mice (129S2-*Tnfrsf1b*^{tm1Mwm}/J). All mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

During the experiment, mice were anesthetized by intraperitoneal injection of α-chloralose (0.07 mg/g) and urethane (1 mg/g); the supplemental doses of α-chloralose and urethane (~1/10 of the initial doses) were injected intravenously to maintain abolition of pain reflexes elicited by paw pinch. The trachea was cannulated, and mice were breathing spontaneously via a tracheal cannula. The jugular vein and femoral artery were cannulated for administering chemicals and for measuring blood pressure (ABP), respectively. Body temperature was maintained at ~36°C by a heating pad. At the end of the experiment, the animals were euthanized by intravenous injection of KCl.

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Intratracheal instillation of TNF-α or vehicle. Mice were anesthetized with inhalation of 2% isoflurane via a nose cone connected to a vaporizer (Protech international model 61020-WBB, Boerne, TX). A small (~0.5 cm) midline incision was made on the ventral neck skin to expose the trachea. Under sterile conditions, TNF-α solution (10 μg/ml; 0.03 ml) or its vehicle (PBS, 0.03 ml) was instilled into the trachea via a needle (28-gauge). After the incision was closed, the mice were allowed to recover from anesthesia, and experiments were carried out at different time points (1–2 or 6–7 days) after the instillation.

Pulmonary chemoreflex experiment. Respiratory flow was measured with a heated mouse pneumotachograph (dead space: 40 μl; airflow resistance: 0.018 $cmH_2O \cdot ml^{-1} \cdot s^{-1}$; linear flow range: 0–3 ml/s; designed and manufactured by the University of Kentucky Center for Manufacturing) connected to the tracheal cannula and a differential pressure transducer (Validyne MP45-14, Northridge, CA). Tidal volume (V_T), expiratory duration (T_e), respiratory frequency (f), and minute ventilation ($\dot{V}_E$) were analyzed on a breath-by-breath basis by an online computer (Biocybernetics TS-100, Taipei, Taiwan). ABP and heart rate (HR) were measured by a pressure transducer (Statham P23AC, Hato Rey, Puerto Rico) and also analyzed by the online computer. The sampling rate was set at 1,000 Hz in this study. Results analyzed by computer were routinely compared with those by hand calculation for accuracy.

Pulmonary chemoreflex responses were elicited by bolus intravenous injections of capsaicin (Cap; a TRPV1 activator; 0.25–1.00 μg/kg), phenylbiguanide (PBG; a 5-HT_3 activator; 30–120 μg/kg), or allyl isothiocyanate (AITC; a TRPA1 activator; 0.3–0.9 mg/kg).

Analyses of airway inflammatory cells and mediators. One day after intratracheal instillation of TNF-α or vehicle, mice were anesthetized by intraperitoneal injection of α-chloralose and urethane as described earlier, and BALF was obtained by injecting a total of 1.1 ml of PBS (lavage twice consecutively at 0.6 ml and 0.5 ml) via the trachea cannula. The collected BALF was centrifuged at 1,500 rpm, 4°C for 10 min. The pelleted cells were treated with 0.24 ml Tris-buffered ammonium chloride solution (pH 7.2) to lyse red blood cells. The remaining cells were washed with 1.2 ml PBS supplemented with Hank's buffer solution with 20% fetal bovine serum (FBS). Total cell counts were determined by using a hemocytometer. Differential leukocyte counts were carried out using cytospin and standard morphologic criteria. The cytospin procedure was performed by University of Kentucky Clinical Laboratory. A minimum of 500 leukocytes were counted by two different individuals, and the data were then averaged.

To compare the inflammatory mediators and cytokines released in the BALF, supernatants were transferred to other tubes for ELISA of inflammatory mediators and cytokines including leukotriene (LT) B_4, $LTC_4/D_4/E_4$, histamine, prostaglandin E_2 (PGE_2), thromboxane B_2 (TXB_2), pentraxin 3 (PTX3), interleukin (IL)-1β and IL-13. The assay of the same chemical in the BALF collected from different treatment groups was performed at the same time to avoid any possible experimental error and variability. These inflammatory mediators and cytokines were selected for measurements based on the previous reports indicating their possible changes in response to the TNF-α treatment (7, 37, 38, 42).

Experimental protocols. Four study series were performed. For Study 1, two matching groups of WT mice were pretreated with intratracheal instillation of PBS and TNF-α, respectively, as described above. Chemoreflex responses were tested and compared between these two groups 24 h later; the protocol was designed based on our preliminary observations. In Study 2, to determine the long-term effect of the TNF-α treatment, chemoreflex responses were tested and compared between three matching groups of WT mice receiving different protocols: *1*) 1–2 days after PBS; *2*) 1–2 days after TNF-α; *3*) 6–7 days after TNF-α; PBS and TNF-α were administered (intratracheal instillation) in an identical manner. In Study 3, to determine whether TNF-α pretreatment induced airway inflammation, BALF was obtained for measurements of inflammatory cells and mediators from five groups of mice 24 h after intratracheal instillation of either PBS or TNF-α as follows: *1*) naïve group (WT mice receiving no surgery or treatment); *2*) Veh group [WT mice pretreated with vehicle (PBS, 0.03 ml)]; *3*) and *4*) TNF-α 1 day group and 7 day group, respectively [WT mice 1 and 7 days after pretreatment with TNF-α (10 μg/ml, 0.03 ml), respectively]; and *5*) p55/p75-null + TNF-α group [p55/p75-null mice pretreated with TNF-α (10 μg/ml, 0.03 ml)]. In Study 4, to investigate the role of TNFRs, pulmonary chemoreflex responses to Cap, PBG, and AITC were compared between WT mice and p55/p75-null mice 24 h after pretreatment with TNF-α. In Study 5, the relative roles of TNFR1 and TNFR2 in the TNF-α-induced hypersensitivity were further determined by comparing the reflex responses to Cap and PBG between p55-null and p75-null mice.

Statistical analysis. Data were reported as means ± SE and analyzed using one-way and two-way repeated-measures ANOVA, unless mentioned otherwise. Pairwise comparisons for ANOVA were made with a post hoc analysis (Fisher's least significant difference). A value of $P < 0.05$ was considered significant.

When the apneic responses were compared between the four groups of mice (WT, p55-null, p75-null, and p55/p75-null), a linear mixed model with the between-subjects factor (group) and within-subjects factors (dose of Cap or PBG) was constructed. The covariance structure of the repeated measurements within the same subject was unstructured since the compound symmetry assumption made by a conventional two-way ANOVA procedure was not supported by the data. A post hoc comparison of means was based on the interaction between the factors group and dose.

Chemical agents. TNF-α was purchased from Prospec Bio (Rehovot, Israel). ELISA kits for $LTC_4/D_4/E_4$, PGE_2, LTB_4, TXB_2 and histamine were purchased from Cayman chemical company (Ann Arbor, MI), PTX3 and IL1-β from R&D systems (Minneapolis, MN), and IL-13 from eBioscience (San Diego, CA). Isoflurane was purchased from Bulter animal health supply (Dublin, OH). Hank's buffer solution and FBS were purchased from Gibco (Grand Island, NY). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

RESULTS

Study 1: pulmonary chemoreflex responses potentiated by TNF-α pretreatment. We found no significant difference in any of the baseline respiratory and cardiovascular variables (V_T, f, $\dot{V}_E$, ABP, and HR) between the two groups of mice 1 day after they received pretreatments with vehicle (Veh) and TNF-α ($P > 0.05$, $n = 6$; Table 1). However, TNF-α pretreatment significantly potentiated the pulmonary chemoreflex responses; representative examples are shown in Fig. 1. Bolus (intravenous) injection of a low dose of Cap (0.5 μg/kg) in a mouse pretreated with vehicle caused only mild cardiorespiratory depression (Fig. 1), whereas the same Cap challenge caused distinct pulmonary chemoreflex responses, characterized by a long apnea accompanied by abrupt and transient decrease in HR and ABP, in a mouse pretreated with TNF-α (Fig. 1). When the apneic response was standardized by calculating the apneic ratio as apneic duration/20-breath average of base-line T_e, the group data showed that the apneic responses to Cap injections were dose dependent in the Veh group (Fig. 2). In comparison, the same Cap challenges elicited significantly larger apneic responses to Cap in the TNF-α-treated group in the entire dose range, except at the lowest dose of 0.25 μg/kg. Furthermore, a potentiating effect of the TNF-α pretreatment with similar pattern and magnitude was also found in the pulmonary chemoreflex responses to intravenous injections of PBG and AITC, as shown in Figs. 1 and 2.

Table 1. *Comparison of baseline respiratory and cardiovascular variables after TNF-α and vehicle pretreatments in anesthetized mice*

	Frequency (bpm)	Tidal Volume (ml)	Ventilation (ml/min)	Blood Pressure (mmHg)	Heart Rate (bpm)
Veh	160.1 ± 6.4	0.115 ± 0.012	18.13 ± 1.72	87.70 ± 9.49	491.7 ± 22.0
TNF-α	158.6 ± 7.4	0.098 ± 0.006	15.35 ± 1.49	85.76 ± 6.20	473.9 ± 31.4
P	0.884	0.224	0.252	0.868	0.651

Values are means ± SE; $n = 6$. Tumor necrosis factor-alpha (TNF-α) group: intratracheal instillation of TNF-α (10 μg/ml, 0.03 ml); vehicle (Veh) group: intratracheal instillation of vehicle (PBS, 0.03 ml).

Study 2: chronic effects of TNF-α pretreatment on pulmonary chemoreflex. In our pilot experiments, we found that the potentiating effect of TNF-α on pulmonary chemoreflex was present in anesthetized mice when tested within 10–100 min after the intratracheal instillation. However, the effect was not as pronounced as 24 h later, and was further masked by an irregular and unstable baseline breathing pattern, presumably resulting from the acute effects of intratracheal instillation. In addition, in a small number of mice ($n = 3$), we also found that the potentiating effect of TNF-α pretreatment was still clearly present after 3–5 days, but began to decline. Hence this study series was intended to determine if the effect of TNF-α persisted 6–7 days after the instillation. Our results showed that the apneic reflex responses to Cap and PBG clearly increased when tested on either 1 or 2 days after the TNF-α pretreatment; because there was no statistically significant difference ($P > 0.1$; paired *t*-test) in the responses between 1 day ($n = 3$) and 2 days ($n = 3$), these data were pooled (Fig. 3). The enhanced apneic response declined significantly toward the baseline at 6–7 days after the instillation, which were no longer different from that in the Veh group (Fig. 3, *left*; $n = 6$). The augmented apneic response to PBG also showed similar but less consistent recovery at 6–7 days; it declined significantly at the high dose (120 μg/kg), but was not significantly different from that at 1–2 days at the medium (60 μg/kg) and low doses (30 μg/kg) of PBG (Fig. 3, *right*; $n = 6$).

Study 3: airway inflammation induced by TNF-α pretreatment. Differential cell count in the BALF showed that the total number of leukocyte cells was significantly higher in the WT mice ($n = 5$) 1 day after a pretreatment with TNF-α than that in the other four groups as follows: naïve group ($P < 0.05$, $n = 5$); Veh group ($P < 0.05$, $n = 5$); TNF-α (7-day) group ($P < 0.05$, $n = 6$); and p55/p75-null + TNF-α group ($P < 0.05$, $n = 5$). No significant difference was found between these four other groups (Table 2). The percentages of eosinophils and neutrophils in the TNF-α (1-day) group were >15-fold higher than that in naïve, Veh, TNF-α (7-day), and p55/p75-null + TNF-α groups (Table 2), and again, no significant difference was found in either eosinophils or neutrophils between these four other groups (Table 2). Although the percentages of lymphocytes and monocytes were lower in the TNF-α (1-day) group, the total numbers of these cells were higher than the other four groups.

ELISA results showed that TNF-α pretreatment significantly elevated the levels of LTB_4, $LTC_4/D_4/E_4$, histamine, TXB_2, and IL-1β in the BALF of WT mice, compared with that in the naïve, Veh, and p55/p75-null groups, and there was no significant difference in these inflammatory mediators and cytokine between these three other groups (Fig. 4; notice that TXB_2 was not measured in naïve animals). In contrast, there was no difference in the level of PGE_2 and IL-13 in BALF among all the groups. The levels of PTX3 were distinctly higher in both TNF-α and Veh groups than that in the naïve group, but there was no difference between TNF-α and Veh groups.

Study 4: the role of TNF-α receptors in the potentiating effect of TNF-α. We found no significant difference in any of the baseline respiratory and cardiovascular variables between WT mice and p55/p75-null mice 1 day after TNF-α pretreatment ($P > 0.05$, $n = 6$ each; Table 3). However, after the TNF-α pretreatment, the pulmonary chemoreflex responses to Cap, PBG, and AITC were clearly stronger in WT mice than that in the p55/p75-null mice, as shown by the representative examples in Fig. 5. Group data further showed that apneic responses to these chemical activators of bronchopulmonary

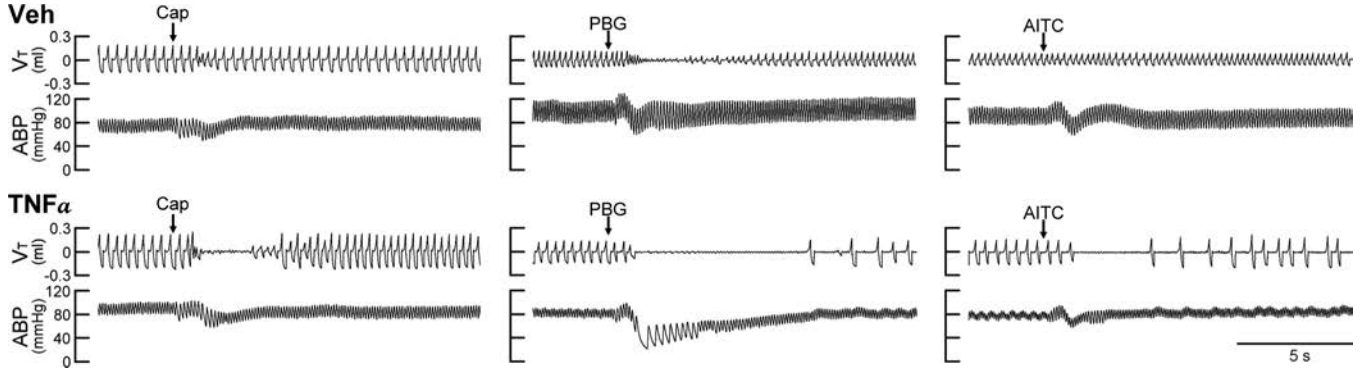

Fig. 1. Experimental records illustrating the effect of tumor necrosis factor-alpha (TNF-α) pretreatment on pulmonary chemoreflexes elicited by capsaicin (Cap), phenylbiguanide (PBG), and allyl isothiocyanate (AITC) in anesthetized spontaneously breathing mice. Treatments were made 24 h before the experiments; Veh: intratracheal instillation of vehicle (PBS, 0.03 ml); TNF-α: intratracheal instillation of TNF-α (10 μg/ml, 0.03 ml). Intravenous bolus (0.05 ml volume) injections of Cap (0.5 μg/kg), PBG (60 μg/kg), and AITC (0.6 mg/kg) were made at the arrows. Body weights in the Veh group (*top*) from left to right were 35.3, 30.1, and 30.1 g, respectively; TNF-α group (*bottom*) were 34.5, 27.0, and 27.0 g, respectively. V_T, tidal volume; ABP, arterial blood pressure.

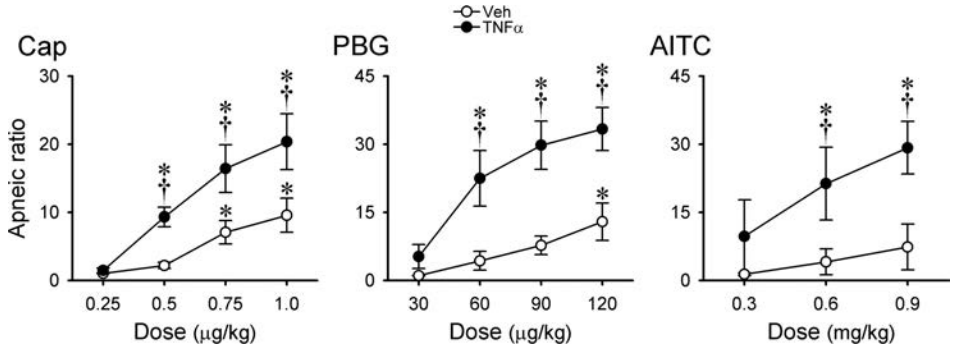

Fig. 2. Dose-related apneic responses to Cap, PBG, and AITC in anesthetized, spontaneously breathing mice after pretreatments with TNF-α and its vehicle. Treatments were made 24 h before the experiments; Veh: intratracheal instillation of vehicle (PBS, 0.03 ml); TNF-α: intratracheal instillation of TNF-α (10 μg/ml, 0.03 ml). Apneic ratio was calculated as (apneic duration/20-breath average of baseline expiratory duration). Responses to Cap, PBG, and AITC were tested in different groups of mice; $n = 6$ in each group (except in the Veh group response to AITC, $n = 5$). Data are means ± SE. *Significantly different from lowest dose in each group ($P < 0.05$); †significantly different from corresponding data in the Veh group ($P < 0.05$).

C-fibers were significantly higher in the WT mice than that in the p55/p75-null mice 24 h after both groups received the TNF-α pretreatment except the lowest doses (Fig. 6). In fact, the cardiorespiratory responses in p55/p75-null mice pretreated with TNF-α were similar to that in the WT mice pretreated with vehicle (PBS) shown in Figs. 1 and 2.

Study 5: the relative roles of TNFR1 and TNFR2 in the potentiating effect of TNF-α. After the TNF-α pretreatment, the increases in apneic responses to 0.5, 0.75, and 1.0 μg/kg Cap in p75-null mice were all significantly smaller than that in the WT mice, but only the response to 1.0 μg/kg was smaller in p55-null mice than that in WT mice (Fig. 7). The TNF-α-induced increases in apneic responses to PBG were significantly attenuated in both p55-null and p75-null mice, compared with that in WT mice, except at the highest dose (120 μg/kg) (Fig. 7). Furthermore, there was no significant difference between p55-null and p75-null mice in the responses of apneic ratio, HR, and ABP to either Cap or PBG.

DISCUSSION

Results of the present study showed that TNF-α instilled into the lung caused an increased sensitivity of pulmonary chemoreflex responses to chemical stimulants of vagal bronchopulmonary C-fiber afferents, including both TRPV1 (Cap) and non-TRPV1 activators (PBG and AITC). This sensitizing effect was found 24 h after the TNF-α treatment, persisted at 48 h, and then gradually declined after several days. The TNF-α-induced hypersensitivity was associated with airway inflammation as shown by striking elevation of the levels of inflammatory cells (neutrophils and eosinophils), autacoids (LTs, histamine), and proinflammatory cytokines (IL-1β) in the BALF. Furthermore, both the increase in pulmonary chemoreflex sensitivity and airway inflammation were completely abrogated in the p55/p75-null mice that were pretreated with TNF-α.

It is well recognized that TNF-α plays an important role in the pathogenesis of chronic inflammatory airway diseases such as asthma. TNF-α is stored in granules and released from mast cells, macrophages, neutrophils, dendritic cells, and other inflammatory cells via the immunoglobulin E-dependent mechanism(s) (4, 16, 20, 42). The proinflammatory effects of TNF-α are mediated through the activation of TNFR1 and TNFR2 (41). Both these TNFRs are present on a wide variety of cell types and are involved in a wide range of immunological responses and inflammatory reactions, but they have distinct

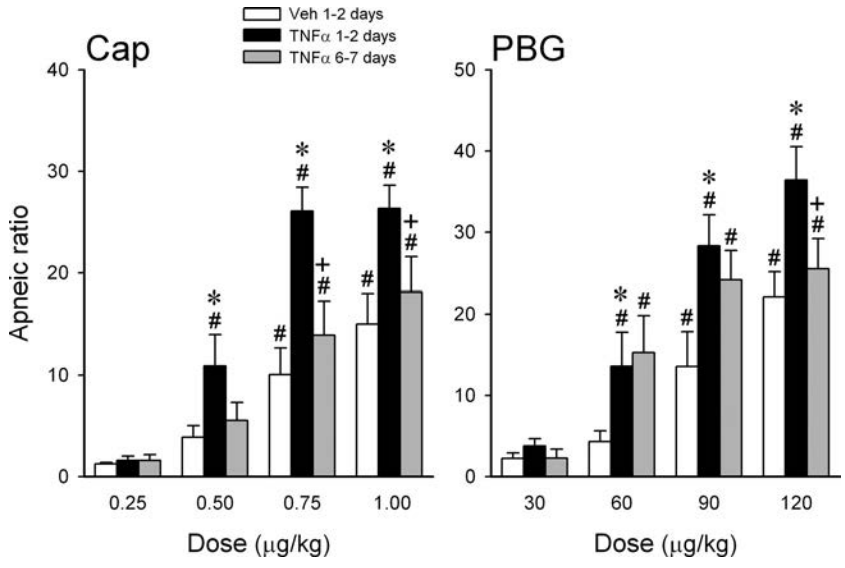

Fig. 3. Chronic effects of TNF-α pretreatment on the apneic responses to Cap and PBG. Measurements were made in 3 different groups of mice, $n = 6$ in each group: *1*) Veh: 1–2 days after intratracheal instillation of vehicle (PBS, 0.03 ml); *2*) and *3*): 1–2 days and 6–7 days after intratracheal instillation of TNF-α (10 μg/ml, 0.03 ml), respectively. Data are means ± SE. *Significantly different from the corresponding response in mice pretreated with vehicle; +significantly different from the corresponding response in mice at 1–2 days after TNF-α pretreatment; #significantly different from the lowest dose in the same group.

Table 2. *Effect of TNF-α pretreatment on leukocyte counts in bronchoalveolar lavage fluid*

Group	Total Cells ($\times 10^5$)	Eosinophils (%)	Neutrophils (%)	Basophils (%)	Lymphocytes (%)	Monocytes (%)
Naïve ($n = 5$)	4.55 ± 1.30	0.98 ± 0.14	0.98 ± 0.27	0.17 ± 0.12	17.14 ± 2.86	80.74 ± 2.54
Veh ($n = 5$)	3.37 ± 1.44	1.21 ± 0.27	1.16 ± 0.20	0.17 ± 0.11	16.38 ± 1.56	81.08 ± 1.42
TNF-α 1 day ($n = 5$)	10.35 ± 3.24*	15.99 ± 1.11*	34.27 ± 2.55*	0.35 ± 0.06	9.91 ± 0.54*	39.47 ± 3.64*
TNF-α 7 days ($n = 6$)	3.79 ± 1.13	0.38 ± 0.03	0.44 ± 0.05	0.13 ± 0.05	5.95 ± 1.35*	93.10 ± 1.35*
p55/p75-null + TNF-α ($n = 5$)	4.24 ± 0.61	0.30 ± 0.06	0.58 ± 0.11	0.16 ± 0.07	7.79 ± 1.78*	91.17 ± 1.82*

Values are means ± SE. Naïve group: wild-type (WT) mice receiving no surgery or treatment; Veh group: WT mice pretreated with vehicle (PBS); TNF-α 1 and 7 days groups: WT mice 1 and 7 days after pretreatment with TNF-α, respectively; p55/p75-null + TNF-α group: p55/p75-null mice pretreated with TNF-α. Both vehicle and TNF-α were delivered by intratracheal instillation. Bronchoalveolar lavage fluid was collected at 24 h after pretreatment with TNF-α or vehicle (except the TNF-α 7 days group). *Significantly different from corresponding data in the naïve group ($P < 0.05$)

molecular masses (55 kDa and 75 kDa), immunoreactivity, and activation mechanisms (8, 41). Binding of TNF-α to these receptors activates certain intracellular signaling pathways that lead to a wide array of cellular responses; for example, activation of TNFR1 can activate nuclear factor-kappa B pathway and mitogen-activated protein kinase pathways, including the extracellular signal-regulated kinase (ERK) (1–3, 15). Activated ERK is then translocated to the nucleus and can alter the gene expression, which has been implicated in the TNF-α-induced overexpression of TRPV1 in nociceptive neurons (19, 44). In addition, activation of TNFRs can also exert a chemotactic action, upregulate the leukocyte-endothelial cell adhesive molecules E-selectin and vascular cell adhesion molecule-1, enhance the production of T_h2 cytokines, and cause infiltration and degranulation of these inflammatory cells in the airways (6, 21, 26, 42, 43). Some of the autacoids released from degranulation of these inflammatory cells may in turn generate a secondary sensitizing effect on bronchopulmonary C-fiber endings and augment the pulmonary chemoreflex responses (31, 32). Whether these signaling pathways and mechanisms are involved in the sensitizing effect of TNF-α on C-fiber afferents observed in this study remains to be determined. Our results showed that the TNF-α-induced increase in pulmonary chemoreflex sensitivity was partially abolished in both p55-null and p75-null mice, and was completely abrogated in p55/p75 mice (Figs. 6 and 7). Furthermore, there was no significant difference in the apneic response to either Cap or PBG between p55-null and p75-null mice, indicating that both types of TNFRs were involved in the sensitizing effects of TNF-α.

To investigate a possible involvement of inflammatory mediators, we selected several mediators for measurements in this study because previous reports indicated their possible releases in the airways resulting from the action of TNF-α (7, 37, 38, 42). Our ELISA data showed distinct increases in LTB_4, $LTC_4/D_4/E_4$, histamine, and TXB_2 in the BALF samples obtained from TNF-α-treated animals, compared with that in the naïve (untreated and non-operated) and vehicle-treated animals (Fig. 4). The cysteinyl LTs, $LTC_4/D_4/E_4$, also known as the slow-reacting substances of anaphylaxis, are produced in cells expressing LTC_4 synthase such as eosinophils and mast cells. These LTs are potent bronchoconstrictors and major contributors to the pathophysiology of asthma, and can induce symptoms of bronchoconstriction, increased vascular permeability, and mucus secretion, etc. (5, 27). Furthermore, it has been reported that these endogenously released lipoxygenase prod-

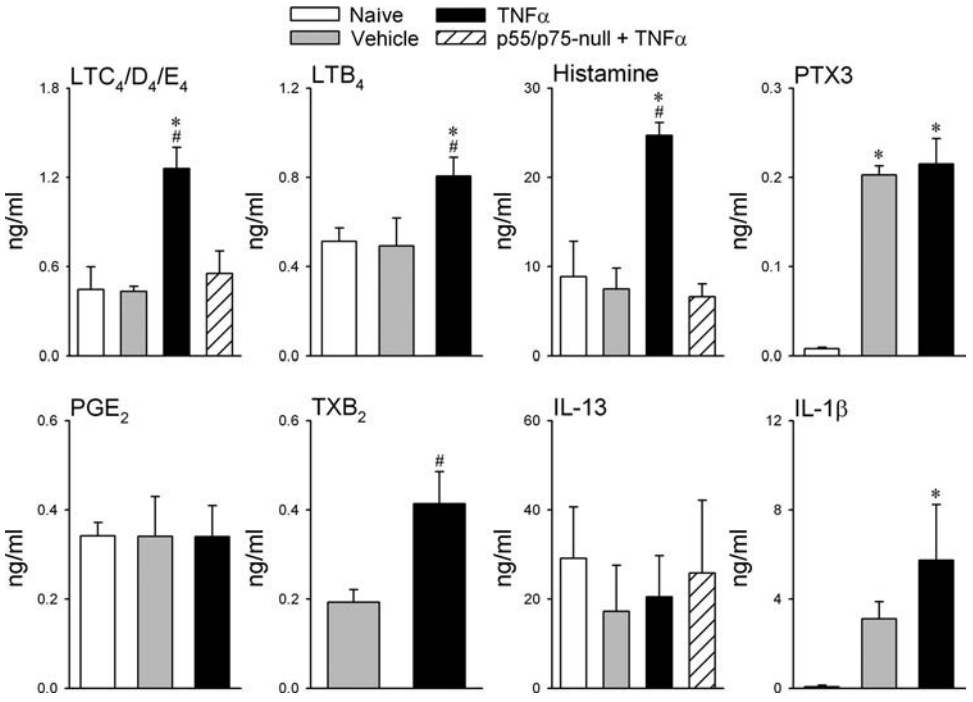

Fig. 4. Effect of TNF-α pretreatment on inflammatory mediators in bronchoalveolar lavage fluid (BALF). Naïve group: wild-type (WT) mice receiving no surgery or treatment; Veh group: WT mice pretreated with vehicle (PBS); TNF-α group: WT mice pretreated with TNF-α; p55/p75-null + TNF-α group: p55/p75-null mice pretreated with TNF-α. Both vehicle and TNF-α were delivered by intratracheal instillation. BALF was collected 24 h after pretreatment of TNF-α or vehicle. LTB_4 and $LTC_4/D_4/E_4$: leukotrienes B_4 and $C_4/D_4/E_4$, respectively; PTX3: pentraxin 3; PGE_2: prostaglandin E_2; TXB_2, thromboxane B_2; IL-13 and IL-1β: interleukins 13 and 1β, respectively. Each data point represents means ± SE of the measurements made in 5 mice; the total number of mice used in these groups varied: $n = 15$ in both Veh and TNF-α groups, $n = 10$ in naïve group, and $n = 5$ in p55/p75-null + TNF-α group. Thus, some tests (e.g., LTB_4, IL-1β) were only performed in 2 or 3 groups of mice. *Significantly different from naïve group ($P < 0.05$); #significantly different from Veh group ($P < 0.05$).

Table 3. *Comparison of baseline respiratory and cardiovascular variables in anesthetized wild-type and p55/p75-null mice*

Group	Frequency (bpm)	Tidal Volume (ml)	Ventilation (ml/min)	Blood Pressure (mmHg)	Heart Rate (bpm)
Wild type	154.3 ± 6.8	0.108 ± 0.005	16.58 ± 1.36	81.07 ± 7.75	537.0 ± 39.3
p55/p75-null	161.1 ± 10.4	0.100 ± 0.010	15.83 ± 0.99	75.26 ± 4.05	504.5 ± 38.1
P	0.594	0.470	0.667	0.522	0.566

Values are means ± SE; $n = 6$. Both groups of mice were pretreated with intratracheal instillation of TNF-α (10 μg/ml, 0.03 ml) 24 h before measurements.

ucts can activate the TRPV1 receptor expressed in the nociceptors in peripheral tissues (22, 24, 40). LTB_4 is produced and released from neutrophils and other leukocytes, is a potent chemoattractant for other inflammatory cells, and can induce the formation of reactive oxygen species by these cells (10, 18). Histamine is mainly produced, stored, and released from the granules of mast cells and basophils upon immunological and chemical actions, and can cause significant bronchoconstrictive and other cardiovascular effects (14). In addition, histamine has been shown to enhance the baseline activity and excitability of bronchopulmonary C-fiber afferents (30, 32). TXB_2 is a stable and inactive metabolite of TXA_2; the latter is a potent prostanoid produced mainly by platelets and known to cause bronchoconstriction and vasoconstriction. TXA_2 has also been shown to activate pulmonary afferents and elicit chemoreflex responses (39). TNF-α instillation into the lung also induced a marked increase in the production of IL-1β in the BALF. IL-1β is produced by macrophages and other inflammatory cells, and has been shown to play a prominent role in the development of airway inflammation and hyperresponsiveness in bronchial asthma (11). A stimulatory effect of IL-1β on the nociceptors in the lung, including pulmonary C-fiber and Aδ afferents, has been reported (17, 46).

PTX3 is a member of the pentraxin superfamily of proteins that are involved in acute immunological response to tissue injury (34). PTX3 is known to be rapidly produced and released by neutrophils and other phagocytes in response to inflammatory signals generated by cytokines such as TNF-α (35). Thus we reasoned that the intratracheal instillation of TNF-α should cause an increase in PTX3 in the BALF. However, we were surprised that no difference in the level of PTX3 was found between the BALF obtained from TNF-α- and vehicle-treated mice, and both PTX3 levels were distinctly higher than that obtained from naïve animals (without surgery or any treatment; Fig. 4). One possible explanation is that the PTX3 released in response to the surgical wound and needle puncture on the trachea during the intratracheal instillation is far greater than the tissue inflammation/injury caused specifically by the TNF-α challenge in this study.

A previous study in our laboratory reported that prolonged incubation (24–48 h) of isolated pulmonary sensory neurons with TNF-α only enhanced their sensitivity to TRPV1 and TRPA1 activators, and not to non-TRP activators of these neurons (21). However, in the present study TNF-α pretreatment augmented the sensitivity of reflex responses not only to Cap, but also to PBG (Figs. 1 and 2); PBG, a selective activator of $5\text{-}HT_3$ receptor, is not known to activate any of the TRP channels. We believe that the discrepancy between these two studies is related to the fact that TNF-α administered in vivo induced leukocyte infiltration and endogenous release of mediators in the lungs in these animals (Table 2 and Fig. 4). As described earlier, some of these inflammatory mediators can enhance the sensitivity of bronchopulmonary C-fiber afferents and pulmonary chemoreflex responses to chemical stimulants such as PBG (12, 21, 31, 32), though we did not attempt to identify any specific mediators that are primarily responsible in this study. Indeed, the potentiated pulmonary chemoreflex responses induced by the TNF-α treatment were absent in p55/p75-null mice (Figs. 4–6) when the accompanying airway inflammatory reactions were abrogated in these mice. Furthermore, the finding that the chemoreflex hypersensitivity subsided when the inflammatory reaction recovered 7 days after the TNF pretreatment in WT mice (Table 2 and Fig. 3) seems to support this hypothesis. However, these new findings in the present study do not rule out the possibility suggested by Hu and co-workers (21) that TNF-α alone can induce a sensitizing effect and/or overexpression of the TRPV receptors in the airway C-fiber sensory neurons.

Morphological and neurophysiological evidence clearly show that vagal C-fiber afferents innervate all levels of the

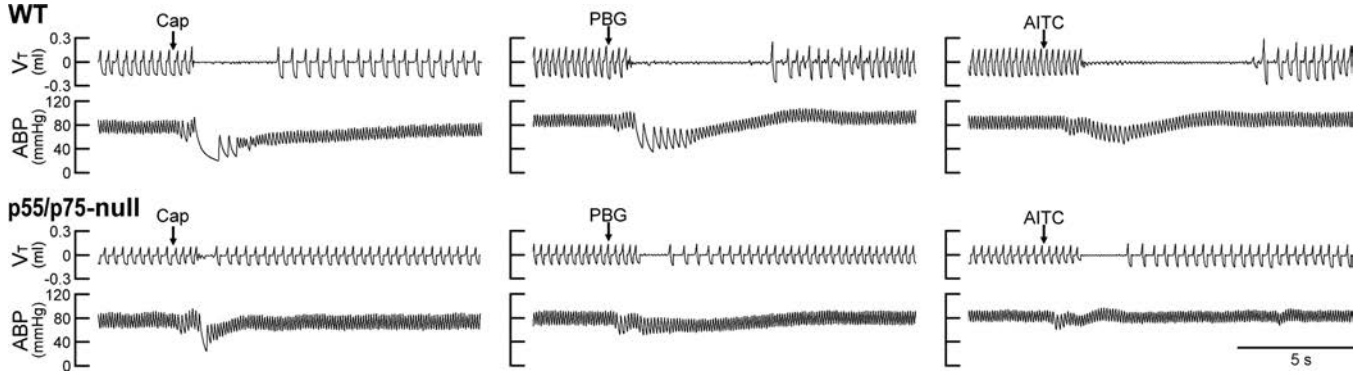

Fig. 5. Experimental records illustrating the effect of TNF-α pretreatment on pulmonary chemoreflexes elicited by Cap, PBG, and AITC in anesthetized WT and p55/p75-null mice. TNF-α (10 μg/ml, 0.03 ml) was administered by intratracheal instillation 24 h before the experiments. Intravenous bolus (0.05 ml volume) injections of Cap (0.5 μg/kg), PBG (60 μg/kg), and AITC (0.6 mg/kg) were made at arrows. Body weights in WT mice (*top*) from left to right were 32.1, 34.5, and 34.5 g, respectively; p55/p75-null mice were 28.3, 26.6, and 26.6 g, respectively.

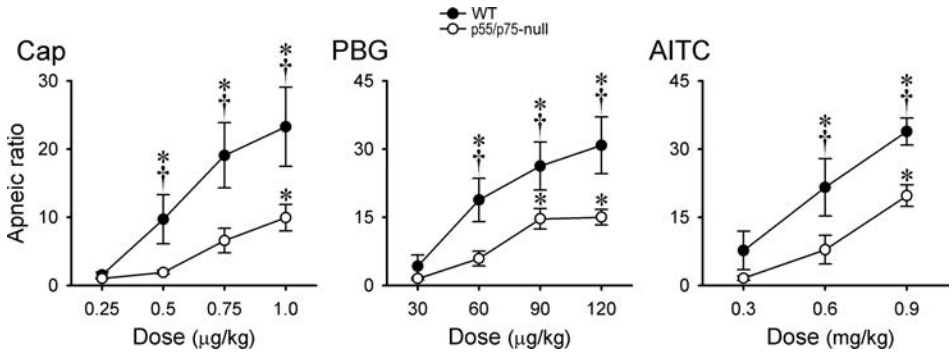

Fig. 6. Effects of TNF-α pretreatment on the apneic responses to Cap, PBG, and AITC in anesthetized WT and p55/p75-null mice. TNF-α (10 μg/ml, 0.03 ml) was administered by intratracheal instillation 24 h before the experiments. Data are means ± SE ($n = 6$). *Significantly different from lowest dose in each group ($P < 0.05$); †significantly different from the corresponding data in p55/p75- null mice ($P < 0.05$).

respiratory tract, from trachea to alveoli, in various mammalian species including humans (12, 31, 32, 45), and they represent the majority of the sensory nerves arising from the lung structures (23). It is well recognized that the afferent activity generated by these C-fiber endings plays an important role in eliciting the pulmonary defense reflexes against the assaults by various chemical irritants in the respiratory tract (12, 31). When these afferent endings are activated, action potentials conducted through the vagus nerves to the commissural subnucleus of the nucleus tractus solitarius elicit the pulmonary chemoreflex response as illustrated in this study (12, 31). Other responses include airway irritation, urge to cough, and dyspnea (9, 29). In addition, intense and/or sustained stimulation of these afferents can cause airway smooth muscle contraction, mucous hypersecretion, vasodilation, and extravasation of macromolecules in the tracheobronchial tree (12, 32). These airway responses are known to be mediated through both the cholinergic reflex pathways and the local "axonal reflex"; the latter involves the release of several bioactive tachykinins and calcitonin gene-related peptides from these sensory endings (13, 33). Although we did not measure these other responses in this study, it seems logical to postulate that when the excitability of C-fiber afferents is enhanced by endogenous TNF-α, these airway and vascular responses to a given stimulus will be amplified.

In conclusion, results of this study showed that intratracheal instillation of TNF-α induced an inflammatory reaction as evidenced by the infiltration of inflammatory cells and releases of potent bronchoactive autacoids in the airways. These effects were accompanied by a pronounced and sustained elevation of pulmonary chemoreflex responses, which was mediated through an activation of both types of TNFRs. Taken together, these results suggest that the hypersensitivity of bronchopulmonary C-fiber afferents may contribute, at least in part, to the airway hyperresponsiveness associated with the increasing level of TNF-α found in airway inflammatory diseases.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

R.-L.L., Y.-J.L., and M.J.G. performed experiments; R.-L.L., R.K., Y.-J.L., M.J.G., and L.-Y.L. analyzed data; R.-L.L., R.K., and L.-Y.L. interpreted results of experiments; R.-L.L. and L.-Y.L. prepared figures; R.-L.L. and L.-Y.L. drafted manuscript; R.-L.L., Y.-J.L., and L.-Y.L. approved final

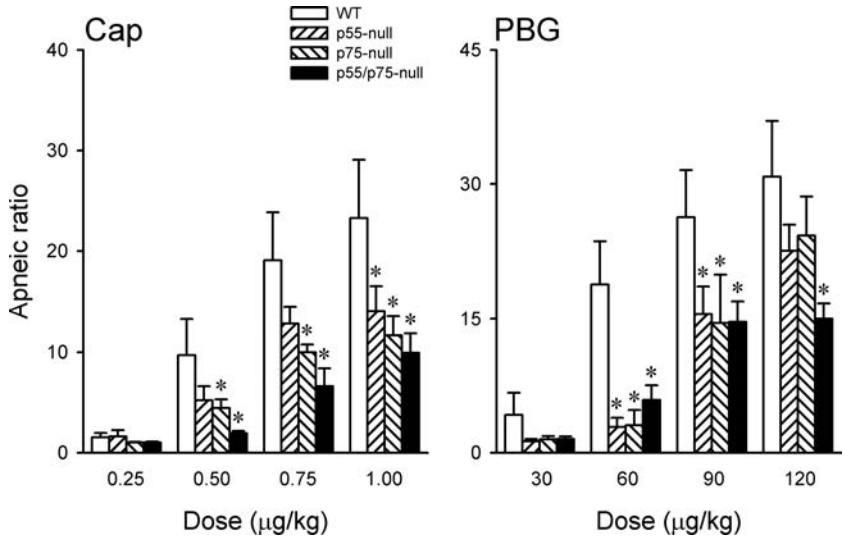

Fig. 7. Relative roles of TNFR1 and TNFR2 in the effects of TNF-α pretreatment on the apneic responses to Cap and PBG. TNF-α (10 μg/ml, 0.03 ml) was administered by intratracheal instillation 24 h before the experiments. Measurements were made in 4 different groups of mice: WT, p55-null (TNFR1 deletion), p75-null (TNFR2 deletion), and p55/p75-null; $n = 6$ in each group. Data from WT and p55/p75 groups were the same as those presented in Fig. 6. Data are means ± SE ($n = 6$). *Significantly different from WT group; since the interaction hypothesis is detected with reduced statistical power, $P < 0.1$ was considered significant in the post hoc analysis.

version of manuscript; L.-Y.L. conception and design of research; L.-Y.L. edited and revised manuscript.

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Hydrogen sulfide induces hypersensitivity of rat capsaicin-sensitive lung vagal neurons: role of TRPA1 receptors

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Hsu CC, Lin RL, Lee LY, Lin YS. Hydrogen sulfide induces hypersensitivity of rat capsaicin-sensitive lung vagal neurons: role of TRPA1 receptors. *Am J Physiol Regul Integr Comp Physiol* 305: R769–R779, 2013. First published July 10, 2013; doi:10.1152/ajpregu.00202.2013.—The sensitization of capsaicin-sensitive lung vagal (CSLV) afferents by inflammatory mediators is important in the development of airway hypersensitivity. Hydrogen sulfide (H_2S) is an endogenous mediator inducing hyperalgesia through transient receptor potential ankyrin 1 (TRPA1) receptors located on nociceptors. We conducted this study to determine whether H_2S elevates the sensitivity of rat CSLV afferents. In anesthetized, artificially ventilated rats, the inhalation of aerosolized sodium hydrosulfide (NaHS, a H_2S donor) caused no significant changes in the baseline activity of CSLV afferents. However, the afferent responses to right atrial injection of capsaicin or phenylbiguanide and to lung inflation were all markedly potentiated after NaHS inhalation. By contrast, the inhalation of its vehicle or NaOH (with a similar pH to NaHS) failed to enhance the afferent responses. Additionally, the potentiating effect on the afferent responses was found in rats inhaling L-cysteine (a substrate of H_2S synthase) that slowly releases H_2S. The potentiating effect of NaHS on the sensitivity of CSLV afferents was completely blocked by pretreatment of HC-030031 (a TRPA1 receptor antagonist) but was unaffected by its vehicle. In isolated rat CSLV neurons, the perfusion of NaHS alone did not influence the intracellular Ca^{2+} concentration but markedly potentiated the Ca^{2+} transients evoked by capsaicin. The NaHS-caused effect was totally abolished by HC-030031 pretreatment. These results suggest that H_2S induces a nonspecific sensitizing effect on CSLV fibers to both chemical and mechanical stimulation in rat lungs, which appears mediated through an action on the TRPA1 receptors expressed on the nerve endings of CSLV afferents.

lung; lung vagal C fibers; afferent sensitization; H_2S; TRPA1 receptors

CAPSAICIN-SENSITIVE LUNG VAGAL (CSLV) afferents are nociceptive-like free nerve endings innervating all levels of the respiratory tract. CSLV afferents can detect several inhaled irritants (22, 25) and inflammatory mediators (16, 25, 26, 39) that might in turn trigger various respiratory reflexes such as cough, mucus secretion, and bronchoconstriction (7, 22). The afferents are sensitized by several mediators released because of lung inflammation (2, 7, 15, 27), which might then exaggerate these respiratory reflexes. Therefore, the sensitization of CSLV afferents is probably involved in the pathogenesis of airway hypersensitivity in diseases such as chronic cough and asthma (7, 26, 47).

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Hydrogen sulfide (H_2S), an irritant gas with the smell of rotten eggs, is emitted principally from volcanoes, hot springs, and numerous industrial sites. Lung exposure to H_2S might lead to adverse respiratory effects, such as cough, airway irritation, airway hypersensitivity, and lung inflammation (16, 37, 41). Therefore, for the past decades, H_2S was generally considered only an exogenous irritant. However, beginning with a report in 1996, H_2S was suggested to act as an endogenous neuromodulator facilitating the hippocampal long-term potentiation in rats (1). Since then, H_2S has been recognized as a signaling molecule involved in a number of physiological processes, such as vasodilation (17), neuronal protection from oxidative stress (20), the regulation of insulin release (49), and the facilitation of bladder contraction (35). Recently, H_2S has been suggested as a proinflammatory mediator under various pathophysiological conditions such as hind paw inflammatory edema (50) and endotoxin-induced systemic inflammation (3, 24).

The application of sodium hydrosulfide (NaHS, a donor of H_2S) has been demonstrated to induce the sensitization of colon nociceptive neurons (44, 48), a counterpart of CSLV neurons in visceral tissues. Moreover, one study showed that the intratracheal instillation of NaHS triggered airway neurogenic inflammation that was nearly abolished by the desensitization of CSLV afferents (41), which implied the effects of H_2S on the afferents. However, whether H_2S acts on CSLV afferents is unknown; if it does, then the H_2S-induced effect and its underlying mechanism remain to be explored. The application of NaHS has been reported to induce somatic hyperalgesia and the depolarization of dorsal root ganglion neurons, which are dependent on transient receptor potential ankyrin 1 (TRPA1) receptors (2, 28, 48). The TRPA1 receptor, a Ca^{2+}-permeable, nonselective cation channel, is predominantly expressed on nociceptive sensory neurons that include CSLV neurons (31, 40). From this information, we conducted this study *1*) to investigate whether H_2S sensitizes CSLV afferents to chemical and mechanical stimulation by using a single-fiber recording technique in anesthetized, artificially ventilated rats; *2*) to determine whether the H_2S-induced effect is also present in isolated CSLV neurons by using a Ca^{2+} image technique; and *3*) to delineate the role of the TRPA1 receptors in H_2S-induced sensitization.

METHODS

The following procedures were performed in accordance with the recommendations found in the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Taipei Medical University.

In Vivo Study

Male SD rats (weighing 320–420 g) were initially anesthetized with an intraperitoneal injection of α-chloralose (100 mg/kg) and urethane (500 mg/kg) dissolved in a borax solution (2%). Supplemental doses of these anesthetics were administrated intravenously to sustain the elimination of pain reflexes produced by pinching the rat's tail throughout the experiment. For the application of anesthetics and pharmacological agents, the left jugular vein was cannulated and a catheter was advanced until its tip was positioned near the right atrium. The right femoral artery was cannulated to measure the arterial blood pressure. Body temperature was maintained at ~36°C throughout the experiment by using a servo-controlled heating pad. At the end of the experiment, the animals were euthanzed using an intravenous injection of KCl.

Measurement of activity of CSLV afferents. Fiber activity arising from CSLV afferents was recorded in the anesthetized, artificially ventilated rats by using the techniques described in our previous studies (14, 25–27). In brief, the trachea was cannulated and tracheal pressure was measured via a sideport of the cannula. The rats were artificially ventilated with a respirator (683, Harvard, South Natick, MA); the tidal volume and respiratory frequency were set at 8 ml/kg and 50 breaths/min, respectively. The expiratory outlet of the respirator was placed under 3- cmH_2O pressure after the chest was opened to identify the locations of the sensory nerve endings. The right cervical vagus nerve was separated and placed on a small dissecting platform. A thin filament was teased away from the desheathed nerve trunk and placed on a platinum-iridium hook electrode. To search for these afferent fibers, the lungs were hyperinflated in a stepwise manner to 3 or 4 tidal volume; the CSLV fibers are activated by lung inflation at this high volume level (22). The thin filament was split until the afferent activity arising from a single unit was electrically isolated. Once the presence of a single unit was detected, capsaicin (1.5 μg/kg) was injected intravenously into the right atrium. Only afferent fibers that met the following criteria were studied: *1*) fibers with a short latency (<2 s) and intense response to the capsaicin injection, and *2*) fibers where the general locations of the receptors could be identified by their responses to the gentle pressing of the lungs with a wet cotton swab at the end of the experiment.

Experimental protocols. Rats were divided into 13 groups to conduct 6 series of experiments. Each group contained 8 rats, and only one CSLV fiber was tested in each rat. The H_2S was delivered by its donor NaHS. To minimize the systemic effect, NaHS solution (5 mg/ml) was provided by aerosol generated through a vibrating plate nebulizer (Aeroneb Pro; Aerogen Nektar, Galway, Ireland). The nebulizer was connected to the breathing circuit between the inspiratory outlet of the respirator and the tracheal cannula. The particle sizes of the aerosol were ~2.1 μm, and the solution volume delivered from nebulizer over 3 min was ~0.25 ml under the experimental conditions. For each NaHS inhalation, the bolus injections of chemical stimulants of CSLV fibers or lung inflations were performed 15 min before and 5 and 30 min after the termination of the inhalation. In *series 1*, to determine the sensitizing effect of H_2S on CSLV fibers, the afferent responses to capsaicin (a selective stimulant of C fibers; 0.5–1.0 μg/kg) were compared between before and after airway exposure to NaHS or its vehicle (saline) in a group of rats (*group 1*). The inhalation of NaHS and its vehicle was performed in alternate order between fibers to achieve a balanced design, and 80 min elapsed between the 2 inhalations for recovery. In *series 2*, to determine whether the H_2S-induced sensitizing effect on CSLV fibers was limited to capsaicin as a stimulant, 2 other stimulants of the fibers were chosen [phenylbiguanide (3–8 μg/kg) (*group 2*) and lung inflation (tracheal pressure = 30 cmH_2O for 10 s) (*group 3*)]. The protocol of this study series was the same as that of *series 1*, except that the stimulant was replaced by phenylbiguanide injection or lung inflation. In *series 3*, because alkalinity has been reported to induce hyperalgesia in mice through TRPA1 receptors (13), to determine whether the H_2S-induced sensitization of CSLV fibers resulted from the alkaline nature of the NaHS solution, the afferent responses to capsaicin (*group 4*) or lung inflation (*group 5*) were compared between the airway exposure to NaHS and its vehicle (saline) in the same group of rats; the pH value of the later was adjusted to 11.3 by adding NaOH (0.1 N). In *series 4*, because the effect of H_2S generated from NaHS (a fast releasing donor) on inflammation was reported to be opposite to that produced from slow releasing donors (45), to determine whether CSLV fibers were also sensitized by slow-releasing agents of H_2S, L-cysteine (a metabolic precursor of H_2S) was chosen as a slow-releasing agent of H_2S (43). The afferent responses to capsaicin (*group 6*) or lung inflation (*group 7*) were compared between the airway exposure to L-cysteine (5 mg/ml for 3 min) and its vehicle (saline) in the same group of rats. *Series 5* was used to evaluate the role of TRPA1 receptors in the H_2S-induced sensitization of the CSLV fibers. As controls, either capsaicin injection or lung inflation was performed before and 5 and 30 min after the NaHS inhalation in two different groups. Subsequently, the experiments were repeated 15 min after pretreatment with HC-030031 (an antagonist of TRPA1 receptors; 8 mg/kg iv) (*groups 8* and *9*) or its vehicle (*groups 10* and *11*). The dose of HC-030031 was shown to exert a complete blocking effect on the stimulation of the CSLV fibers elicited by the TRPA1 receptor agonist in our previous study (25). The purpose of *series 6* was to verify the effectiveness and specificity of the antagonizing effect of HC-030031 on the TRPA1 receptors under the present experimental conditions. The sensitizing responses of the CSLV afferents to capsaicin were compared before and after HC-030031 pretreatment. Protocols same to *series 5* were used, except that the fibers were sensitized using allyl isothiocyanate (AITC, a selective agonist of TRPA1 receptors; 0.4 $mg \cdot kg^{-1} \cdot min^{-1}$, 2 min) (*group 12*) and using prostaglandin E_2 (PGE_2; 3 $\mu g \cdot kg^{-1} \cdot min^{-1}$, 2 min) (*group 13*). The PGE_2 has been demonstrated to induce hypersensitivity of CSLV afferents in our previous study (21).

In Vitro Study

In addition to its primary expression on the sensory neurons, TRPA1 receptors are also expressed on nonneuronal cells in the lung (12, 32). Mediators released by these nonneuronal cells after NaHS exposure might lead to a secondary sensitizing effect on the in vivo preparation of CSLV afferents (15, 22, 25, 26). To determine whether H_2S exerts a sensitizing effect on the CSLV neurons, the following experiments were performed in vitro preparation.

Labeling CSLV neurons with DiI. Sensory neurons innervating the lungs and airways were identified by retrograde labeling from the lungs with the fluorescent tracer 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), as described previously (14, 18, 21). In brief, young male SD rats (~150 g) were anesthetized by aerosolized isoflurane (2% in O_2) through a nose cone connected to a vaporizing machine (AM Bickford, New York City, NY). To expose the trachea, a small midline incision was made on the ventral neck skin. The DiI (0.2 mg/ml; 0.05 ml) was instilled into the lungs through a needle (30 gauge) inserted into the trachea lumen, and the incision was then closed. To allow the DiI to be transported toward the soma of CSLV neurons, the animals were kept undisturbed for 7–10 days until they were euthanized for the cell culture.

Isolation and culture of nodose and jugular ganglion neurons. The methodology was described in detail in our previous studies (14, 18, 21). Male SD rats were anesthetized with 5% isoflurane and were decapitated. The head was quickly immersed in an ice-cold DMEM/F-12 solution, followed by the extraction of the nodose and jugular ganglia. Each ganglion was desheathed, cut, placed into a mixture of type IV collagenase (0.04%) and dispase II (0.02%), and incubated for 80 min in 5% CO_2 in air at 37°C. The ganglion suspension was centrifuged (150 *g*, 5 min), and the supernatant was aspirated. The pellet was then resuspended in a modified DMEM/F12 solution and gently triturated. The dispersed cell suspension was centrifuged (500

g, 8 min) through a layer of bovine serum albumin (15%) to separate the cells from the myelin debris. The pellets were resuspended in the modified DMEM/F12 solution plated onto poly-L-lysine-coated glass coverslips and were then incubated overnight (5% CO_2 in air at 37°C).

Measurement of Ca^{2+} transients. Cells were washed or maintained with an extracellular solution in a small-volume (0.2 ml) perfusion chamber at room temperature. The Ca^{2+} transients were measured in these cells by using a Zeiss digital fluorescence microscope (Axiovert 100; Carl Zeiss, Thornwood, NY) equipped with a variable filter wheel (Sutter Instruments, Novato, CA) and a digital CCD camera (Princeton Instruments, Trenton, NJ), as previously described (14, 18). Before the Ca^{2+}-imaging experiments, cells were incubated with 5 μM Fura-2 acetoxymethyl ester for 30 min at 37°C, then rinsed with an extracellular solution, and allowed to deesterify for at least 30 min before use. Dual images (340 and 380 nm excitation, 510 nm emission) were collected, and the pseudocolored ratiometric images were monitored during the experiments. The imaging system was standardized with a 2-point calibration by using a Ca^{2+}-free standard (−) and a Ca^{2+}-saturated standard (+). Both standards contained 11 μM Fura-2 [44 μl of 10 mM Fura-2 penta K^+ salt, 8 ml of 20 mM sodium HEPES (pH 7.4), and 32 ml H_2O] and were prepared as follows: − standard, 18 ml Fura-2 and 1.98 ml of 10 mM sodium EGTA (pH 7.6); and + standard, 18 ml Fura-2 and 1.98 ml of 10 mM $CaCl_2$. The parameters used for the 2-point calibration included the dissociation constant of Fura-2 (K_d 285), the ratio values for the (−) and (+) concentration standards (R_{min} and R_{max}, respectively) and the denominator wavelength intensities for the (−) and (+) concentration standards (Den_{min} and Den_{max}, respectively). The intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$, in nM) was calculated according to the following equation: $[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R)(Den_{min}/Den_{max})$. Typical R_{min} and R_{max} values were 0.225 and 1.45, respectively.

Experimental protocols. After the incubation period with Fura-2 AM, the coverslip containing the cells was mounted into a chamber placed on the stage of the microscope. The entire chamber was continuously perfused with an extracellular solution during the experiment by a gravity-fed valve-controlled system (VC-66CS, Warner Instruments, Hamden, CT) at a constant rate of ~2 ml/min. The KCl solution (60 mM, 20 s) was perfused at the end of each experimental run to test for cell viability. The CSLV neurons were selected from the cultured cells for analysis that met the following criteria: *1*) a spherical shape with no neurite outgrowths, *2*) activated by capsaicin (200 nM, 30 s), and *3*) labeled with DiI fluorescence. A total of 112 neurons from 11 rats were studied in three separate series of experiments. *Series 1* was performed to examine the sensitizing effect of H_2S on the CSLV neurons; Ca^{2+} transients elicited by capsaicin (200 nM, 30 s) were determined before and 1 min after the onset of NaHS perfusion (200 μM, 2.5 min). *Series 2* was performed to evaluate the role of the TRPA1 receptors; the NaHS-induced potentiation of capsaicin-evoked Ca^{2+} transients was determined after the pretreatment of HC-030031 (20 μM, 16.5 min). *Series 3* was performed to investigate whether the inhibitory effect of HC-030031, if any, on the potentiation of Ca^{2+} transients resulted from its suppressive action on capsaicin. The Ca^{2+} transients evoked by capsaicin alone were determined before and after HC-030031 pretreatment.

Pharmacological agents. In the in vivo study, a stock solution of capsaicin (250 μg/ml) was prepared in 1% Tween 80, 1% ethanol, and 98% saline; and a stock solution of phenylbiguanide (400 μg/ml) was prepared in saline. The solutions of capsaicin and phenylbiguanide for injection at the desired concentrations were prepared daily by dilution with saline based on the animal's body weight. A stock of HC-030031 (30 mg/ml) was dissolved in dimethyl sulfoxide and further diluted to a final concentration of 2 mg/ml with a vehicle (10% Tween 80, 10% ethanol, and 80% saline) before use. In the in vitro study, desired concentrations of the pharmacological agents were prepared in a similar manner, except that the extracellular solution, instead of saline, was used as the vehicle. An extracellular solution was prepared with 5.4 mM KCl, 136 mM NaCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.33 mM NaH_2PO_4, 10 mM glucose, 10 mM HEPES, and a pH level adjusted to 7.4 with NaOH and the osmolarity to 300 mosM. A modified DMEM/F12 solution was prepared using DMEM/F-12 supplemented with a 10% vol/vol heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μM MEM nonessential amino acids. The pH value of the extracellular solution containing NaHS (200 μmol/l) was 7.42. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except HC-030031 (Tocris, Ellisville, MO), dispase II (Roche, Indianapolis, IN), DMEM/F12 (Invitrogen, Carlsbad, CA), and Fura-2 AM and DiI (Molecular Probes, Eugene, OR).

Data Analysis

In the in vivo studies, the fiber activity of CSLV fibers, heart rate, and mean arterial blood pressure were continually analyzed at 1-s intervals over an interval of at least 20 s before and 60 s after the challenges of the chemical or mechanical stimulants. The baseline of these physiological parameters was calculated as the average value over the 10-s period immediately preceding a challenge. The peak response was defined as the maximum 3-s average within 20 s following the injection of the chemical stimulant, or over 5 s after the lung inflation. A fiber was considered activated when the increase in fiber activity exceeded 0.5 impulses/s. These physiological parameters were analyzed using a computer equipped with an A/D converter (DASA 4600, Gould, Columbus, OH) and software (BioCybernatics, 1.0, Taipei, Taiwan). In the in vitro studies, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was continually analyzed at 2-s intervals during the experiments by using the Axon Imaging Workbench software (Axon Instruments, Union City, CA). An increase in intracellular Ca^{2+} concentration ($\Delta[Ca^{2+}]_i$) was measured as the difference between the peak amplitude of Ca^{2+} transients (4 s average) and the 30-s average at baseline. Data were compared using a paired *t*-test or a two-way repeated-measures analysis of variance (ANOVA). When the ANOVA showed a significant interaction, pair-wise comparisons were made with a post hoc analysis (Newman-Keuls test). A value of $P < 0.05$ was considered significant. All data are reported as means ± SE.

RESULTS

In Vivo Study

A total of 104 CSLV afferents were studied in 104 anesthetized, artificially ventilated rats. The locations of the CSLV nerve endings were as follows: 19, 41, 32, and 10 in the upper, middle, lower, and accessory lobes of the right lung, respectively. The locations of the remaining 2 fibers could not be identified. The 104 fibers were divided evenly into 13 groups for the studies in 6 series of experiments.

Effects of NaHS inhalation on baseline CSLV afferent activity. CSLV afferents had a very low and irregular baseline activity during eupneic breathing (Figs. 1 and 2; Table 1). Eighteen of these CSLV fibers had sparse activity, whereas the rest of the 86 were silent under basal condition (Fig. 1). The averaged baseline activity of the CSLV fibers was 0.04 ± 0.01 impulses/s. Five minutes after exposure to NaHS or its vehicle, no significant change was observed in the average baseline activity of these fibers (Fig. 1; Table 1), despite a slight increase in 8 fibers. In addition, the inhalation of NaHS or its vehicle had no detectable effect on the baseline heart rate and mean arterial blood pressure (Fig. 1; Table 1).

Sensitization of CSLV afferents by NaHS. During the control experiments, the injection of low-dose capsaicin abruptly triggered a mild and short burst of discharge in the CSLV affer-

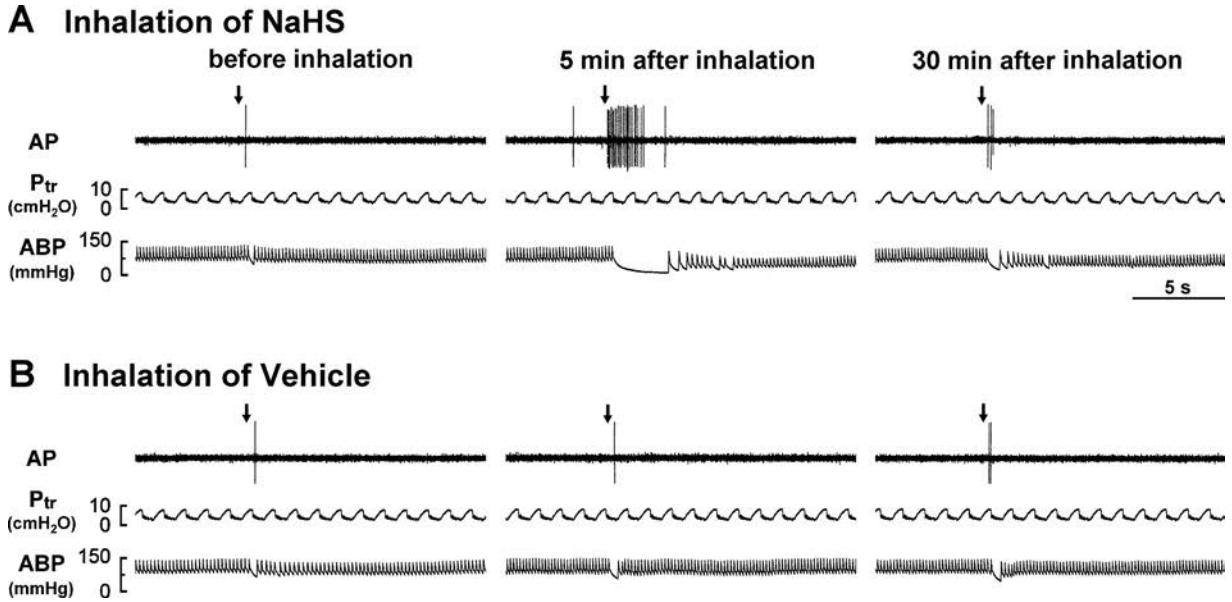

Fig. 1. Experimental records illustrating the responses of a capsaicin-sensitive lung vagal (CSLV) afferent to the right atrial bolus injection of capsaicin (0.75 μg/kg, arrows) before and 5 and 30 min after the termination of airway exposure to aerosolized sodium hydrosulfide (NaHS, a donor of H_2S) in an anesthetized, artificially ventilated rat. *A* and *B*: inhalation of aerosolized NaHS (5 mg/ml, 3 min) and its vehicle (saline), respectively. AP, action potential; P_{tr}, tracheal pressure; ABP, arterial blood pressure. Note that the afferent response to capsaicin was enhanced 5 min after NaHS inhalation but was not affected after vehicle inhalation.

ents (Figs. 1 and 2*A*); the difference between the mean peak response and the baseline fiber activity was 0.74 ± 0.15 impulses/s (Fig. 2*A*). However, the stimulatory effect by the same dose of capsaicin on these fibers was markedly potentiated (5.4-fold relative to its control) 5 min after NaHS inhalation; the response to capsaicin returned to control levels 30 min after inhalation (Fig. 2*A*). In contrast, inhalation of the vehicle did not significantly change the afferent response to capsaicin (Figs. 1 and 2*A*). The potentiating effect of NaHS was not limited only to the response triggered by capsaicin injection. Similarly, the afferent responses (increase in fiber activity: 1.74 ± 0.81 impulses/s) elicited by phenylbiguanide injection were enhanced (3.4-fold relative to its control) 5 min after NaHS inhalation, whereas the inhalation of its vehicle did not have such an enhancing effect (Fig. 2*B*). At 5 min after NaHS inhalation, the fiber responses to these chemical stimulants increased significantly in both the peak activity and the duration of the firing (Figs. 1*A*, and 2, *A* and *B*). The durations of the firing evoked by capsaicin before and 5 min after NaHS inhalation were 2.4 ± 0.8 and 6.3 ± 2.0 s ($n = 8$, $P < 0.05$), respectively. The durations of the firing evoked by phenylbiguanide before and 5 min after NaHS inhalation were 4.0 ± 2.2 and 13.4 ± 2.1 s ($n = 8$, $P < 0.05$), respectively. Furthermore, the potentiating effect of NaHS was found in the afferent response to lung inflation. Before NaHS inhalation (in the control group), the CSLV fibers were relatively insensitive to lung inflation (Fig. 2*C*); only 5 of the 8 CSLV fibers were slightly activated by constant-pressure lung inflation (averaged increase in fiber activity: 0.44 ± 0.23 impulses/s, $n = 8$). At 5 min after inhalation, NaHS significantly potentiated the stimulatory effect of lung inflation (8.5-fold relative to its control), whereas the vehicle failed to do so (Fig. 2*C*). The NaHS-induced potentiation to lung inflation was reversed 30 min after the inhalation (Fig. 2*C*).

NaOH failed to mimic the sensitization of CSLV afferents by NaHS. Five minutes after the inhalation of an aerosolized NaOH solution with a similar pH (value = 11.3) to NaHS, the baseline fiber activity of the CSLV fibers did not change significantly (before NaOH: 0.02 ± 0.01 impulses/s, 5 min after NaOH: 0.04 ± 0.02 impulses/s; $n = 16$, $P > 0.05$). Similarly, NaOH inhalation did not affect the afferent responses to chemical stimulation provided by capsaicin injection (Fig. 3*A*). In addition, NaOH inhalation did not affect the afferent responses to lung inflation (Fig. 3*B*). However, in the same group of CSLV fibers, NaHS still exerted a markedly sensitizing effect as shown previously (Fig. 3).

CSLV afferents were also sensitized by L-cysteine. Five minutes after the inhalation of an aerosolized L-cysteine solution, the baseline fiber activity of the CSLV fibers did not change significantly (before L-cysteine: 0.04 ± 0.02 impulses/s, after L-cysteine: 0.03 ± 0.02 impulses/s; $n = 16$, $P > 0.05$). However, L-cysteine enhanced the afferent responses to chemical stimulation by capsaicin injection (Fig. 4*A*) and by lung inflation (Fig. 4*B*). The sensitizing effect of L-cysteine returned to control levels within 90 min after inhalation (Fig. 4). In contrast, inhalation of the vehicle did not significantly change the afferent responses (Fig. 4).

Role of TRPA1 receptors in the sensitization of CSLV afferents by NaHS. The pretreatment of HC-030031 did not alter the baseline fiber activity of the CSLV afferents. As a group, in the control experiments, the baseline fiber activities before and after HC-030031 pretreatment were 0.02 ± 0.01 and 0.03 ± 0.03 impulses/s ($n = 16$; $P > 0.05$), respectively. Nevertheless, the HC-030031 pretreatment completely blocked the NaHS-induced potentiating effect on the fiber responses to capsaicin injection (Figs. 5 and 6*A*) and to lung inflation (Fig. 6*C*). Pretreatment with the HC-030031 vehicle did not affect the potentiating effect caused by NaHS on the fiber response to either capsaicin injection (Fig. 6*B*) or lung inflation (Fig. 6*D*).

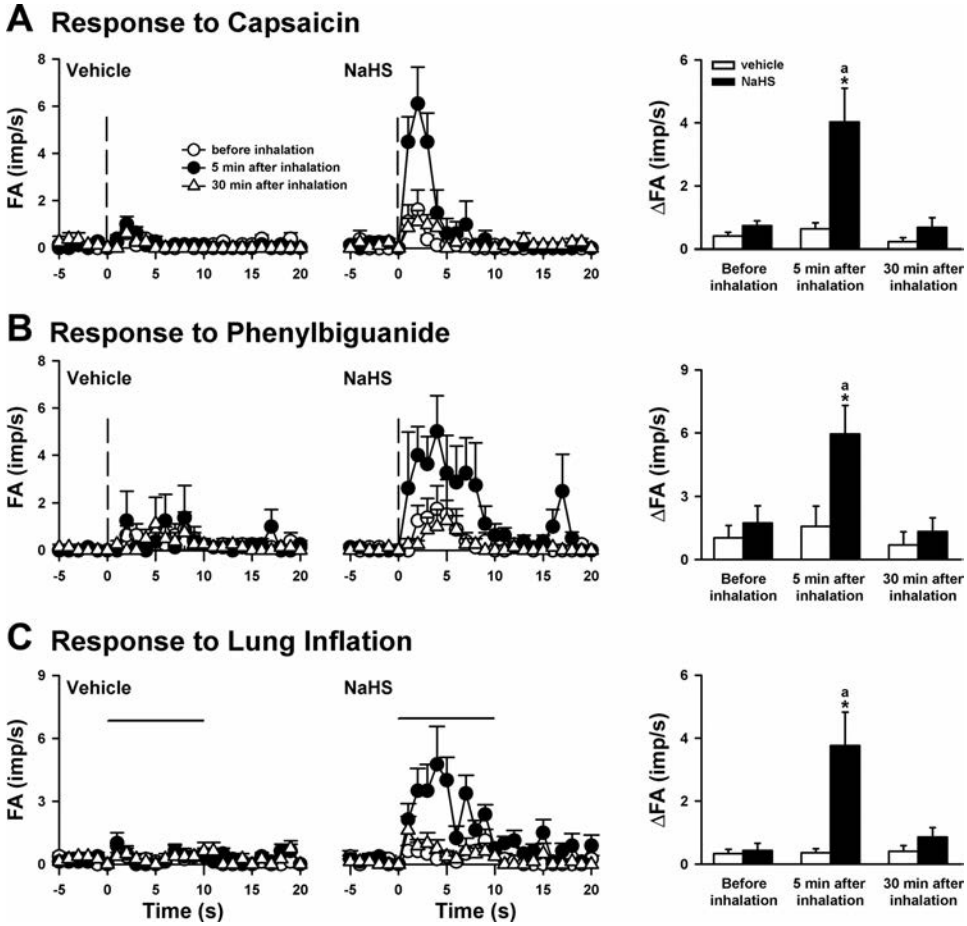

Fig. 2. The afferent responses of CSLV fibers to the right atrial bolus injection of capsaicin (*A*) and phenylbiguanide (*B*) and to lung inflation (*C*) before and 5 and 30 min after the termination of airway exposure to aerosol in 3 groups of rats. *Middle* and *left*: average afferent responses at groups of inhalation of NaHS aerosol (5 mg/ml, 3 min) and its vehicle (saline), respectively. Vertical dashed lines in *A* and *B* are the onset time of the right atrial injection of capsaicin (0.5–1.0 μg/kg) and phenylbiguanide (3–8 μg/kg), respectively. The horizontal lines in *C* are the duration of lung inflation (P_{tr} = 30 cmH_2O, 10 s). FA, fiber activity; imp, impulses. *Right*: average peak afferent responses to inhalation of NaHS aerosol and its vehicle (saline). In *A* and *B*: the increase in fiber activity (ΔFA) was measured as the difference between peak FA (averaged over 3-s intervals) and baseline FA in each fiber. In *C*: ΔFA was measured as the difference between the peak FA (averaged over a 5-s interval) during inflation and the baseline FA. [a]Significantly different from the value before inhalation in the same group ($P < 0.05$). *Significantly different from the value at the corresponding time period in the vehicle group ($P < 0.05$). Data in each group are means ± SE of 8 fibers recorded from 8 rats.

The effectiveness and specificity of the antagonizing effect of HC-030031 were evaluated with AITC (a selective agonist of TRPA1 receptors) and PGE_2 (a sensitizer of CSLV fibers), respectively. Five minutes after AITC infusion, no significant change was noted in the baseline fiber activity of the CSLV fibers (before infusion: 0.03 ± 0.02 impulses/s; after infusion: 0.03 ± 0.03 impulses/s; $n = 8$, $P > 0.05$). However, AITC markedly elevated the excitability of the CSLV fibers to capsaicin (Fig. 6*E*). Pretreatment with HC-030031 completely blocked the hypersensitivity of the CSLV fibers caused by AITC infusion (Fig. 6*E*). In another group of fibers, the blocking effect of HC-030031 on the sensitization of the CSLV fibers was absent if the fibers were sensitized by PGE_2 infusion (Fig. 6*F*).

Table 1. *Effects of inhalation of NaHS and its vehicle on the baselines of CSLV fiber activity, mean arterial blood pressure, and heart rate in anesthetized, artificially ventilated rats*

	Vehicle of NaHS (n = 24)	NaHS (n = 72)
CSLV fiber activity, impulses/s		
Before inhalation	0.05 ± 0.02	0.04 ± 0.01
5 Min after inhalation	0.06 ± 0.02	0.06 ± 0.01
30 Min after inhalation	0.05 ± 0.02	0.04 ± 0.01
Mean arterial blood pressure, mmHg		
Before inhalation	85 ± 5	81 ± 3
5 Min after inhalation	78 ± 5	83 ± 3
30 Min after inhalation	83 ± 5	84 ± 3
Heart rate, beats/min		
Before inhalation	349 ± 14	334 ± 9
5 Min after inhalation	337 ± 16	317 ± 9
30 Min after inhalation	337 ± 15	333 ± 10

Data (means ± SE) are values averaged over 10-s periods before and 5 min and 30 min after the termination of inhalation. *n* represents fiber numbers. Lungs were exposed to aerosolized NaHS (5 mg/ml) or its vehicle for 3 min. Only one capsaicin-sensitive lung vagal (CSLV) fiber was studied in each rat. No statistical significance was found between any 2 groups of mean.

In Vitro Study

In total, 112 isolated CSLV neurons cultured from both nodose and jugular ganglia in 11 rats were studied. The average baseline $[Ca^{2+}]_i$ was 112.3 ± 5.4 nM.

Enhancement of capsaicin-evoked Ca^{2+} transients by NaHS. The application of capsaicin (200 nM, 30 s) evoked a rapid and transient increase in $[Ca^{2+}]_i$ in isolated rat CSLV neurons from both the nodose and jugular ganglia. As shown in Fig. 7*A*, NaHS application alone did not have a detectable effect on the basal $[Ca^{2+}]_i$. In the nodose neurons, the baseline $[Ca^{2+}]_i$ was 105.8 ± 16.6 and 105.6 ± 16.6 nM ($n = 18$, $P > 0.05$) before and 1 min after NaHS perfusion, respectively. In the jugular neurons, the basal $[Ca^{2+}]_i$ was 99.2 ± 9.3 and 99.4 ± 9.7 nM ($n = 24$, $P > 0.05$) before and 1 min after NaHS perfusion, respectively. However, without a detectable effect on baseline $[Ca^{2+}]_i$, the NaHS pretreatment markedly enhanced the peak

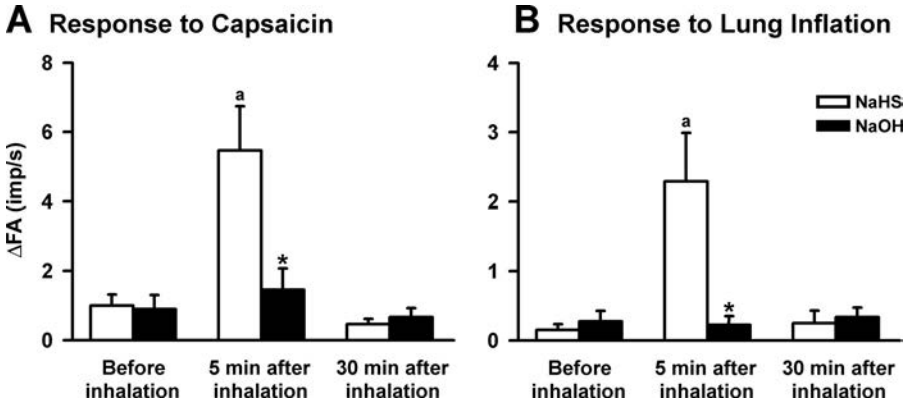

Fig. 3. The effects of inhalations of NaHS and sodium hydroxide (NaOH) on the afferent responses of CSLV fibers to the right atrial bolus injection of capsaicin (*A*) and to lung inflation (*B*) in 2 groups of rats. The NaOH was dissolved in the saline (NaHS vehicle) at pH 11.3 to mimic the pH effect of a NaHS solution. [a]Significantly different from the value before inhalation in the same group ($P < 0.05$). *Significantly different from the value at the corresponding time period in the NaHS group ($P < 0.05$). Data in each group are means ± SE of 8 fibers recorded from 8 rats. See Fig. 2 for further explanation.

Ca^{2+} transients evoked by capsaicin (Figs. 7*A* and 8*A*). The NaHS-induced enhancing effect gradually declined after washout but remained higher than in the control for > 4 min after NaHS application in all tested CSLV neurons (Fig. 7*A*). In addition to its effect on the amplitude, NaHS pretreatment markedly prolonged the duration of the Ca^{2+} transients triggered by capsaicin (Fig. 7*A*). The durations were 11.4 ± 3.3 and 134.4 ± 21.3 s ($n = 18$, $P < 0.05$) in the control and NaHS-treated groups, respectively, in the nodose neurons. The durations were 31.7 ± 12.2 and 172.6 ± 17.3 s ($n = 24$, $P < 0.05$) in the control and NaHS-treated groups, respectively, in the jugular neurons.

Role of TRPA1 receptors in the NaHS-induced enhancement of Ca^{2+} transients. HC-030031 pretreatment did not affect the Ca^{2+} transients evoked by capsaicin alone (300 nM, 30 s) in CSLV neurons, which were 68.4 ± 15.8 and 92.6 ± 22.7 nM ($n = 30$, $P > 0.05$) in the control and HC-030031-pretreated groups, respectively. However, the NaHS-induced enhancement of Ca^{2+} transients evoked by capsaicin was absent if NaHS was applied after the HC-030031 pretreatment (Figs. 7*B* and 8*B*).

DISCUSSION

The results of this study demonstrate that, in anesthetized and artificially ventilated rats, the inhalation of aerosolized NaHS, a donor of H_2S, caused no detectable change in the baseline parameters of CSLV fiber activity or in the mean arterial blood pressure and the heart rate. However, the afferent responses of CSLV fibers to the chemical stimulation by the right atrial bolus injection of capsaicin or phenylbiguanide were all markedly potentiated. The sensitizing effect of NaHS was also found if the CSLV fibers were activated by lung inflation. In addition, the NaHS-induced sensitizing effect on the CSLV fiber response to capsaicin injection or lung inflation was totally abolished by pretreatment with a selective TRPA1 receptor antagonist, HC-030031, but was unaffected by its vehicle. In isolated rat CSLV neurons, we demonstrated that NaHS perfusion did not influence the baseline $[Ca^{2+}]_i$ but significantly potentiated the Ca^{2+} transients evoked by capsaicin. Consistent with our findings from the in vivo electrophysiological experiment, pretreatment with HC-030031 fully reversed the NaHS-induced elevation of Ca^{2+} transients. Taken together, these results suggest that H_2S induces a nonspecific sensitizing effect on CSLV fibers to both chemical and mechanical stimulation in rat lungs, and this effect is mediated, at least in part, through an action on the TRPA1 receptors expressed in the nerve endings of CSLV fibers.

In this study, H_2S was applied by its donor, NaHS. The formation of HS^- from the dissociation of NaHS to $Na^+ + HS^-$ and the subsequent production of H_2S were expected to elevate pH value. An alkaline solution has been shown to induce hyperalgesia in mouse hind paws through an activation of TRPA1 receptors (13). The sensitizing effect of NaHS possibly results from its alkalinity. However, the inhalation of an aerosolized NaOH solution (with a similar pH to NaHS) did not cause any effect on the basal activity and the excitability of the CSLV fibers under our experimental conditions. Therefore, we can rule out the possibility that the alkaline property of NaHS is responsible for

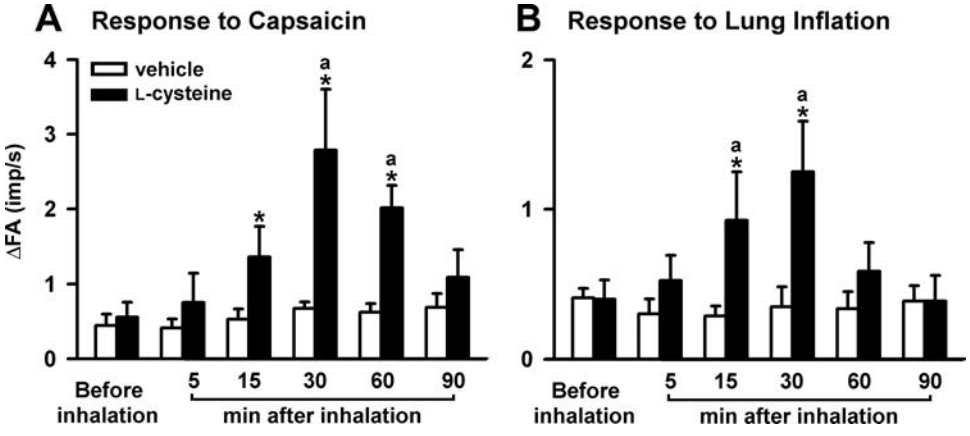

Fig. 4. The afferent responses of CSLV fibers to the right atrial bolus injection of capsaicin (*A*) and to lung inflation (*B*) before and 5, 15, 30, 60, and 90 min after the termination of airway exposure to aerosolized L-cysteine (5 mg/ml for 3 min) in 2 groups of rats. [a]Significantly different from the value before inhalation in the same group ($P < 0.05$). *Significantly different from the value at the corresponding time period in the vehicle group ($P < 0.05$). Data in each group are means ± SE of 8 fibers recorded from 8 rats. See Fig. 2 for further explanation.

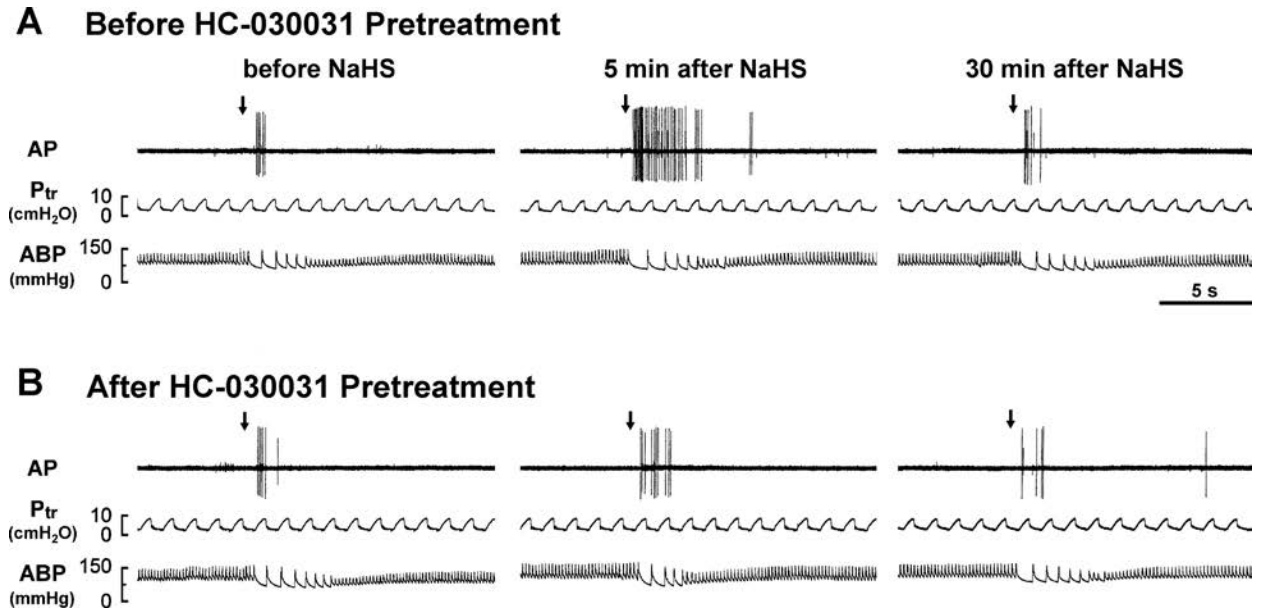

Fig. 5. Experimental records illustrating the effect of pretreatment with HC-030031 on the responses of a CSLV afferent to capsaicin injection (1 μg/kg, arrows) before and 5 and 30 min after the termination of the airway exposure to aerosolized NaHS (5 mg/ml, 3 min) in an anesthetized, artificially ventilated rat. *A* and *B*: before and after pretreatment with HC-030031 (an antagonist of TRPA1 receptors; 8 mg/kg), respectively. Note that pretreatment with HC-030031 blocked the sensitizing effect of NaHS on the fiber response to the capsaicin injection.

the sensitization of CSLV fibers. In addition to NaHS, the sensitizing effect on CSLV fibers was also found in rats pretreated with L-cysteine, which is a common substrate for endogenous H_2S generating enzyme in mammalian cells. However, the exogenous application of L-cysteine could also exert effects independent of H_2S. For example, L-cysteine enters many metabolic pathways, such as metabolism of glutathione, methionine, and coenzyme A (30). The cysteine metabolites are involved in attenuating oxidative stress (4). Thus we cannot totally rule out the possibility that L-cysteine-induced sensitization resulted from the effects of cysteine metabolites.

Several studies have suggested that H_2S is involved in the pathogenesis of airway neurogenic inflammation through an action on the CSLV fibers (5, 42); however, there is no direct evidence of the effect of H_2S on these afferents. Thus this study reported the first evidence that H_2S applied exogenously exerts a sensitizing effect on the CSLV fibers in response to various types of stimuli. Our findings were consistent with other studies that have shown that, in trigeminal ganglia (20) and colonic dorsal root ganglia (48), the exogenous application of H_2S by its donor elevates the excitability of rat nociceptive neurons. Our observations also gain support from the findings that, in temporomandibular joints (11), hind paws (28), and the colon (48), the local application of NaHS induces hyperalgesia, which is a behavioral consequence of the sensitization of nociceptive neurons. In addition to exogenous H_2S, endogenous H_2S has also been suggested to play a vital role in inflammation-induced visceral (11) and somatic (12) hyperalgesia, which is substantiated because the hyperalgesia was reduced by the systemic pretreatment of the inhibitor of H_2S-producing enzymes. However, in these studies, because the enzyme inhibitor was applied systemically, a possibility that the hyperalgesic effect of endogenous H_2S results from its action on the central nervous system but not on peripheral nociceptive nerve endings cannot be ruled out. Thus, whether endogenous H_2S contributes to the sensitization of peripheral nociceptive neurons, including CSLV neurons, is still unknown. Our study provides evidence that, without a significant influence on the basal activity, exogenous H_2S exerts a sensitizing effect on CSLV neurons in the lungs. This finding suggests a potential environmental impact of H_2S, but the role of endogenous H_2S in relation to inflammation of the airways remains to be determined.

We demonstrated that H_2S sensitizes CSLV neurons mediated through the activation of the TRPA1 receptor. In addition to TRPA1 receptors, H_2S has been identified as an endogenous ligand of several receptors/channels that might also participate in nociceptor sensitization and hyperalgesia under various experimental conditions (8, 11, 33, 38). For example, the suppression of sustained potassium channel currents has been suggested to be responsible for the NaHS-induced sensitization of rat trigeminal ganglion neurons innervating the temporomandibular joints (11). It has been shown that the $Ca_v3.2$ T-type Ca^{2+} channels involves the somatic hyperalgesia induced by the intraplantar injection of NaHS (28, 33) and also contributes to the bladder hyperalgesia induced by the intraperitoneal injection of cyclophosphamide (29). Moreover, a recent study concluded that the activation of voltage-gated sodium channels by endogenous H_2S is vital to the sensitization of colonic dorsal root ganglion neurons induced by heterotypic intermittent stress (44). In summary, these results suggest that H_2S-induced hyperalgesia and nociceptive hypersensitivity appear to be related to the contribution of potassium channels, T-type Ca^{2+} channels, and voltage-gated sodium channels. In this study, we suggest that the H_2S-induced sensitization of CSLV neurons relies on the activation of TRPA1 receptors. Our observation is in general agreement with two recent reports that have demonstrated the vital role of TRPA1 receptors in H_2S-induced somatic hyperalgesia (2, 33). The receptors/channels responsible for H_2S-induced hyperalgesia are di-

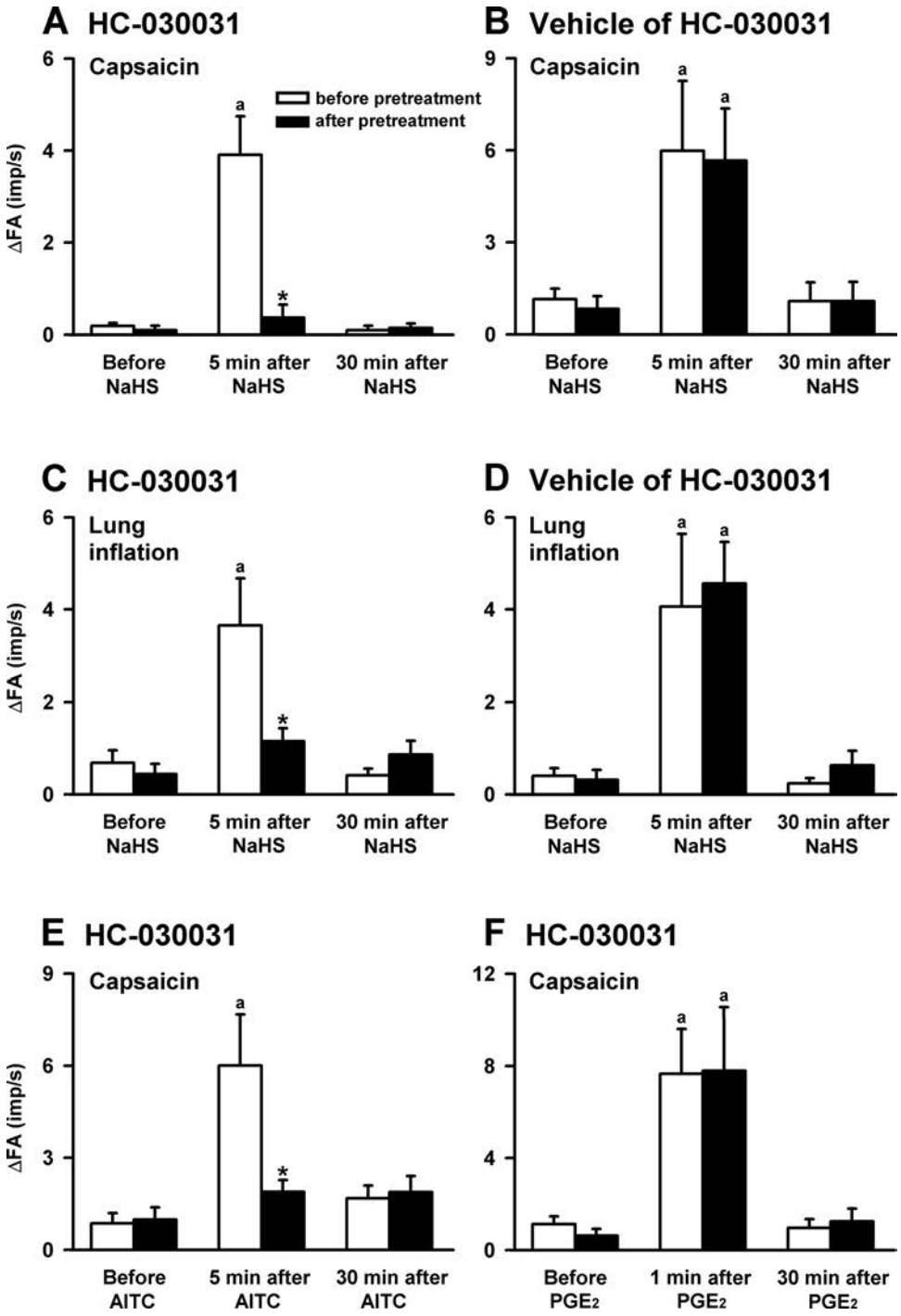

Fig. 6. The effects of pretreatment with HC-030031 on the CSLV-fiber sensitization induced by NaHS, allyl isothiocyanate (AITC), or prostaglandin E_2 (PGE_2) in 6 groups of rats. The NaHS (*A–D*; 5 mg/ml, 3 min) was provided through aerosol inhalation, and AITC (*E*; an agonist of TRPA1 receptors; 0.4 $mg \cdot kg^{-1} \cdot min^{-1}$, 2 min) and PGE_2 (*F*; a sensitizer of CSLV afferents; 3 $\mu g \cdot kg^{-1} \cdot min^{-1}$, 2 min) were applied by intravenous infusion. *Top* and *bottom*: responses to capsaicin injections; *middle*: response to lung inflation. [a]Significantly different from the value before the sensitizing agents in the same group ($P < 0.05$). *Significantly different from the value at the corresponding time period before pretreatment ($P < 0.05$). Data in each group are means ± SE of 8 fibers recorded from 8 rats. See Fig. 2 for further explanation.

verse and might vary with different inducers/locations of the hyperalgesic responses. This notion is supported by a study showing that H_2S-induced hyperalgesia in somatic tissue is dependent on both T-type Ca^{2+} channels and TRPA1 receptors, whereas that in the colon is mediated through T-type Ca^{2+} channels but not through TRPA1 receptors (2). In this study, the significance of TRPA1 receptors was substantiated by the full blockade of HC-030031 pretreatment on the NaHS-induced sensitization. To verify that this conclusion is accurate, we examined the effectiveness and specificity of the antagonizing effect of HC-030031 on TRPA1 receptors under the present experimental conditions. The effectiveness of HC-030031 was demonstrated by its total blockade of CSLV-fiber sensitization induced by AITC, a selective activator of TRPA1 receptors (Fig. 6*E*), whereas the specificity of HC-030031 was determined by its ineffectiveness on CSLV-fiber sensitization caused by PGE_2 (Fig. 6*F*), which sensitizes the fibers through EP prostanoid receptors (21).

The cellular mechanism by which H_2S sensitizes CSLV fibers remains unknown. In this study, the stimulation of the fibers elicited by injection of capsaicin or phenylbiguanide and by lung inflation was all potentiated by H_2S inhalation. Cap-

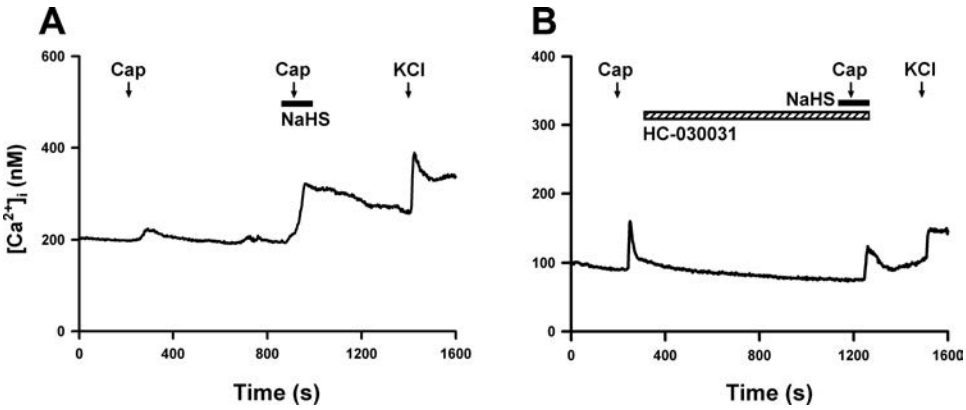

Fig. 7. Experimental records illustrating the potentiating effect of NaHS on capsaicin-evoked Ca^{2+} transients in 2 rat CSLV neurons. *A* and *B*: without and with HC-030031 pretreatment (20 μM, 16.5 min; hatched horizontal bar), respectively. Both neurons were isolated from nodose ganglia. Capsaicin (Cap, 200 nM, 30 s; arrows) was applied 1 min after the onset of NaHS perfusion (200 μM, 2.5 min; filled horizontal bars), and a KCl solution (60 mM, 20 s; arrows) was applied to test the cell vitality at the end of both experiments. $[Ca^{2+}]_i$, intracellular concentration of Ca^{2+}. Note that pretreatment with HC-030031 totally abolished the potentiating effect of NaHS on the Ca^{2+} transients evoked by capsaicin.

saicin and phenylbiguanide are known to be mediated through their activation of the TRP vanilloid 1 (TRPV1) receptor and the serotonin 5-HT_3 receptor, respectively (39), whereas mechanical stimulation of the CSLV fibers possibly involves as yet unidentified cationic channels gated by mechanical stress in the membrane of the nerve terminals (39). Because the afferent responses of CSLV fibers to injection of capsaicin or phenylbiguanide and to lung inflation were all potentiated, this suggests a nonspecific increase in the electrical excitability of CSLV fibers induced by NaHS inhalation. Alternatively, the H_2S-induced activation of TRPA1 receptors probably shares a common cellular mechanism that enhances the function of these chemosensitive and mechanosensitive receptors. Our observations gain support from findings showing that, in rodent dorsal root ganglion neurons, the activation of TRPA1 receptors by AITC enhanced the inward currents evoked by repeated applications of AITC (36) and by the mechanical stimulation of neurites (6). The cellular mechanisms by which TRPA1 activation leads to the sensitization of CSLV fibers is unclear, but they are probably related to changes in the membrane conductance or to the resting membrane potential (19), or both. Activation of TRPA1 receptors evokes the influx of cations, such as calcium and sodium ions, which in turn can trigger the opening of voltage-gated cation channels (19) and lead to elevation of cell excitability of CSLV neurons. In addition to its abundant location on sensory neurons, TRPA1 receptors are also expressed on nonneuronal tissues including in mice and human airways (12, 32). The release of inflammatory mediators caused by the activation of nonneuronal TRPA1 receptors (12, 32), which might then lead to the sensitization of CSLV fibers (7, 22, 39).Furthermore, in mouse airways, H_2S was reported to evoke releases of pro-inflammatory neuropeptides, such as substance P (3, 5). Thus a possibility that the sensitizing effect of H_2S is due to its indirect action on the releases inflammatory mediators should be considered. However, we demonstrated that NaHS-induced sensitization was also found in the isolated rat CSLV neurons, which suggests, at least in part, a direct action of H_2S on the nerve endings of CSLV fibers.

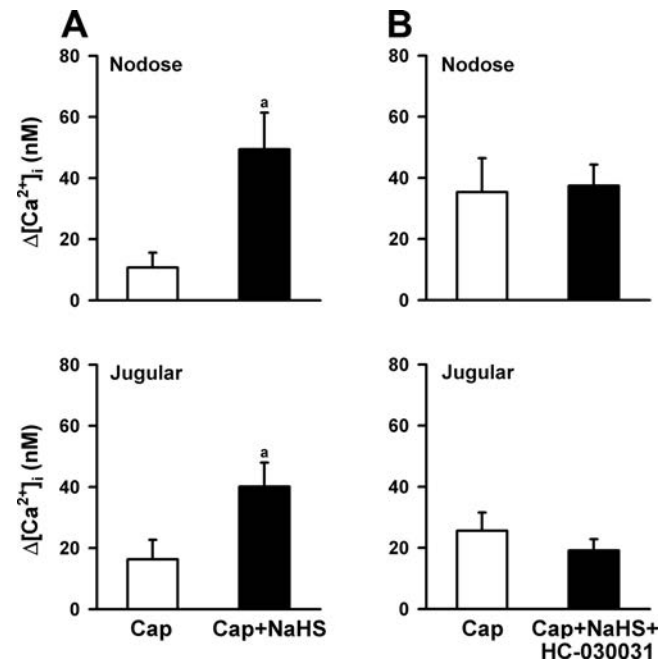

Fig. 8. The effects of HC-030031 pretreatment on the potentiation of capsaicin (Cap)-evoked Ca^{2+} transients by NaHS (200 μM, 2.5 min) in CSLV neurons. *A*: without HC-030031 pretreatment (20 μM, 16.5 min) in nodose ($n = 18$) and jugular ($n = 24$) ganglion neurons. *B*: pretreatment with HC-030031 in nodose ($n = 22$) and jugular ($n = 18$) ganglion neurons. See Fig. 7 for further explanation. An increase in intracellular Ca^{2+} concentration ($\Delta[Ca^{2+}]_i$) was measured as the difference between the peak amplitude of Ca^{2+} transients (4-s average) and the 30-s average at baseline. [a]Significantly different from the value of Cap only ($P < 0.05$). Data in each group are means ± SE.

The elevation of plasma H_2S levels has been reported in certain lung inflammatory conditions, such as in patients with chronic obstructive pulmonary disease (10) and in mice with lipopolysaccharide pretreatment (24). Furthermore, pharmacological inhibition or the genetic ablation of endogenous H_2S-producing enzymes reduces lung inflammation caused by lipopolysaccharides (3, 24). Thus H_2S is believed to act as a pro-inflammatory mediator. However, the role of H_2S in inflammatory reaction remains controversial, with anti-inflammatory effects having been documented (9, 42, 43, 51). The controversy might be related to several factors such as the concentration, the duration of presence, and site of production (15, 46). Regardless of its role in the inflammatory reaction, H_2S exerts a distinct sensitizing effect on CSLV afferents. The concept was supported by our results that sensitizing effect of CSLV-afferents was observed in rats treated with NaHS or L-cysteine; treatments with the former and the latter were reported to produce pro-inflammatory and anti-inflammatory effects, respectively (45). The stimulation of CSLV afferents is well documented to elicit various reflex responses, such as

cough, bronchoconstriction, and airway hypersecretion (7, 22). Once the afferents are sensitized by mediators, this result might in turn exaggerate these respiratory reflexes (7, 15, 23, 26). Accordingly, during lung inflammation, we propose that H_2S might contribute to the pathogenesis of airway hypersensitivity such as chronic cough. However, whether the endogenous H_2S and its sensitizing effect on CSLV afferents play any part in the regulation of airway functions during lung inflammation remains to be determined.

Perspectives and Significance

Our results have demonstrated that in anesthetized and artificially ventilated rats, airway exposure to H_2S causes an increase in the excitability of CSLV afferents. Furthermore, H_2S pretreatment markedly enhanced chemical stimulation-evoked Ca^{2+} transients in isolated rat CSLV neurons. The H_2S-induced sensitizing effect seems to be mediated through an action on TRPA1 receptor, which is probably located at terminals of CSLV fibers. Thus our findings provide new information for understanding of the physiological effects of H_2S in the respiratory tract.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.-C.H. and Y.S.L. conception and design of research; C.-C.H., R.-L.L., and Y.S.L. performed experiments; C.-C.H. and R.-L.L. analyzed data; C.-C.H., L.-Y.L., and Y.S.L. interpreted results of experiments; C.-C.H. and Y.S.L. prepared figures; C.-C.H. and Y.S.L. drafted manuscript; C.-C.H., L.-Y.L., and Y.S.L. edited and revised manuscript; C.-C.H. and Y.S.L. approved final version of manuscript.

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Bronchoconstriction induced by increasing airway temperature in ovalbumin-sensitized rats: role of tachykinins

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[1]*Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan, Republic of China;* [2]*Department of Physiology, College of Medicine, Taipei Medical University, Taipei, Taiwan, Republic of China; and* [3]*Department of Physiology, University of Kentucky Medical Center, Lexington, Kentucky*

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Hsu CC, Lin RL, Lin YS, Lee LY. Bronchoconstriction induced by increasing airway temperature in ovalbumin-sensitized rats: role of tachykinins. *J Appl Physiol* 115: 688–696, 2013. First published July 11, 2013; doi:10.1152/japplphysiol.00491.2013.—This study was carried out to determine the effect of allergic inflammation on the airway response to increasing airway temperature. Our results showed the following: *1*) In Brown-Norway rats actively sensitized by ovalbumin (Ova), isocapnic hyperventilation with humidified warm air (HWA) for 2 min raised tracheal temperature (T_{tr}) from 33.4 ± 0.6°C to 40.6 ± 0.1°C, which induced an immediate and sustained (>10 min) increase in total pulmonary resistance (R_L) from 0.128 ± 0.004 to 0.212 ± 0.013 $cmH_2O \cdot ml^{-1} \cdot s$ ($n = 6$, $P < 0.01$). In sharp contrast, the HWA challenge caused the same increase in T_{tr} but did not generate any increase in R_L in control rats. *2*) The increase in R_L in sensitized rats was reproducible when the same HWA challenge was repeated 60–90 min later. *3*) This bronchoconstrictive effect was temperature dependent: a slightly smaller increase in peak T_{tr} (39.6 ± 0.2°C) generated a significant but smaller increase in R_L in sensitized rats. *4*) The HWA-induced bronchoconstriction was not generated by the humidity delivered by the HWA challenge alone, because the same water content delivered by saline aerosol at room temperature had no effect. *5*) The HWA-evoked increase in R_L in sensitized rats was not blocked by atropine but was completely prevented by pretreatment either with a combination of neurokinin (NK)-1 and NK-2 antagonists or with formoterol, a β_2 agonist, before the HWA challenge. This study showed that increasing airway temperature evoked a pronounced and reversible increase in airway resistance in sensitized rats and that tachykinins released from the vagal bronchopulmonary C-fiber endings were primarily responsible.

vagus; reflex; hyperthermia; extravasation; asthma

BODY TEMPERATURE INCREASES as a result of elevated metabolic rate or hindered heat dissipation. Although the lungs are enclosed in the thoracic chamber and constantly exposed to body temperature, an increase in lung temperature can occur under both normal and pathophysiological conditions. For example, body core temperature exceeding 41°C has been reported in healthy humans and animals during exertional exercise (7, 30). Body temperature higher than 40.5°C occurs frequently in patients suffering from severe fever or heatstroke (5). In addition, tissue inflammation can lead to an increase in local temperature in the inflamed area (16, 38). Indeed, a recent report showed that the end-expiratory temperature plateau was 2.7°C higher in mild allergic asthmatic children than in healthy children, and the difference was closely correlated with the exhaled nitric oxide concentration as well as the sputum eosinophil percentage, suggesting an involvement of local tissue inflammation (37).

A recent study in our lab reported that an increase in intrathoracic temperature to above a threshold of ~39.2°C activated vagal pulmonary C-fiber endings in anesthetized rats (39). A follow-up study further demonstrated a similar stimulatory effect of increasing temperature in isolated rat vagal pulmonary sensory neurons (35). These studies clearly indicated that an increase in temperature within the physiological range can stimulate vagal bronchopulmonary C fibers, and, more importantly, activation of these afferents is known to elicit bronchoconstriction mediated through both cholinergic reflex pathways and local release of tachykinins (10, 21, 24, 28, 41). Indeed, we recently reported that an increase in airway temperature by hyperventilation with hot humid air for 4 min triggered an immediate and transient bronchoconstriction in patients with mild asthma but not in healthy individuals (17). The bronchoconstriction was accompanied by cough and prevented by pretreatment with ipratropium, a muscarinic receptor antagonist, suggesting an involvement of airway sensory nerves. However, the specific type of airway nerves involved could not be determined (17). In a parallel study utilizing an animal model of allergic asthma [Brown-Norway rats actively sensitized by ovalbumin (Ova)], we demonstrated that chronic airway inflammation enhanced the sensitivity of the bronchopulmonary C-fiber afferents to capsaicin, but whether the temperature sensitivity of these afferents was also elevated is not known (47).

To answer these questions, this study was carried out to investigate whether an increase in airway temperature evokes a more intense bronchoconstriction in Ova-sensitized Brown-Norway rats and, if so, to elucidate the possible underlying mechanism(s).

MATERIALS AND METHODS

The procedures described below were performed in accordance with recommendations from the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were also approved by the University of Kentucky Institutional Animal Care and Use Committee.

Animal Sensitization

Young male pathogen-free Brown-Norway rats were divided into two groups (control and sensitized groups). Sensitized animals received an initial intraperitoneal injection of a suspension containing 2 mg of Ova in 1 ml of Imject Alum as adjuvant. Three days later, rats were exposed to Ova aerosol for 15 min three times per week (M/W/F) for 3 wk, following the protocol established by previous investigators (13, 47). During the exposure, the unanesthetized rat was placed in a Plexiglas restrainer (University of Kentucky Center for

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Manufacturing) and breathed spontaneously and continuously through a nose cone connected to a free stream of air-aerosol mixture under a negative-pressure exhaust hood. Ova solution (wt/vol concentration: 1.25% in saline) was nebulized and delivered by an ultrasonic nebulizer (model 099HD; Devilbiss, Somerset, PA) at a droplet size ranging from 0.5 to 5 μm. Control animals received an intraperitoneal injection and aerosol inhalation of the vehicle (isotonic saline), following the identical procedures.

Animal Preparation

One day after the last Ova or saline exposure, animals were anesthetized with an intraperitoneal injection of α-chloralose (100 mg/kg; Sigma-Aldrich, St. Louis, MO) and urethane (500 mg/kg; Sigma-Aldrich) dissolved in a 2% borax solution. During the experiments, supplemental doses of the same anesthetics were injected intravenously to maintain the abolition of the pain reflex elicited by paw pinch. Animals were placed in a supine position and ventilated mechanically with a respirator (model 683; Harvard, South Natick, MA) via a short tracheal cannula inserted just below the larynx after a tracheotomy. Body temperature was maintained at ~36°C by means of a heating blanket placed under the animal. A polyethylene catheter was inserted into the jugular vein until its tip was close to the right atrium for bolus injections of drugs. The right and left femoral arteries were cannulated for measurements of arterial blood gas and arterial blood pressure (ABP), respectively. Respiratory frequency was set at 60 breaths/min, and tidal volume (V_T) was adjusted between 6 and 7 ml/kg in each animal to maintain the end-tidal CO_2 concentration (model 1260; Novametrix, Wallingford, CT) between 4.6% and 5.0%.

Measurement of Lung Mechanics

One day after the last saline or Ova exposure, control and sensitized animals were anesthetized and artificially ventilated in the same manner. A catheter was inserted into the right intrapleural cavity between the fifth and sixth ribs for measuring intrapleural pressure (P_{ip}). Pneumothorax was then corrected by briefly opening the intrapleural catheter to ambient air during a held hyperinflation ($3 \times V_T$). Transpulmonary pressure was measured as the difference between the tracheal pressure and P_{ip} with a differential pressure transducer. Respiratory flow was measured with a heated pneumotachograph and a differential pressure transducer. These signals were analyzed by an online computer (Biocybernetics TS-100, Taipei, Taiwan) for measurements of total pulmonary resistance (R_L), dynamic lung compliance (C_{dyn}), ABP, and heart rate (HR). Results obtained from the computer were routinely checked by hand calculation for accuracy.

Challenge with Humidified Warm Air

The method for generating humidified warm air (HWA) in this study was described previously (26). Briefly, the outlet of the respirator inspiratory line was connected to an air stone that was immersed in isotonic saline contained in a bottle placed in a heated water bath (Fig. 1*A*); isotonic saline was used for generating humidity because inhalation of distilled water or hypotonic saline aerosol has been shown to evoke reflex bronchoconstriction in patients with asthma (40). HWA was then delivered directly into the lung via the tracheal tube. Humidified room air (HRA) was delivered in the same manner except that the water bath was kept at room temperature (~23°C). During HWA or HRA hyperventilation challenges, minute ventilation was increased to ~375% of the baseline (V_T and respiratory frequency were set to 150% and 250% of the baseline, respectively) for 2 min. To prevent hypocapnia and alkalosis produced by hyperventilation, a gas mixture containing 3.2–4.5% CO_2, 21% O_2 with the remaining percentage as N_2 was administrated via the respirator. A miniature thermometer (model IT-18; Physitemp, Clifton, NJ) was inserted into the tracheal tube and positioned near the thoracic entry (~0.5 cm distal to the tip of the tracheal tube) to continuously measure the air temperature in the trachea (T_{tr}; Fig. 1*A*) before, during, and after the HWA and HRA challenges. We determined in our pilot experiments that average T_{tr} can be elevated to ~40.6°C by maintaining the heated water bath temperature at ~70°C (Fig. 1*A*). The amount of water content in HWA and HRA measured in this study was 77.6 ± 5.9 and 15.2 ± 0.3 mg/l of air, respectively.

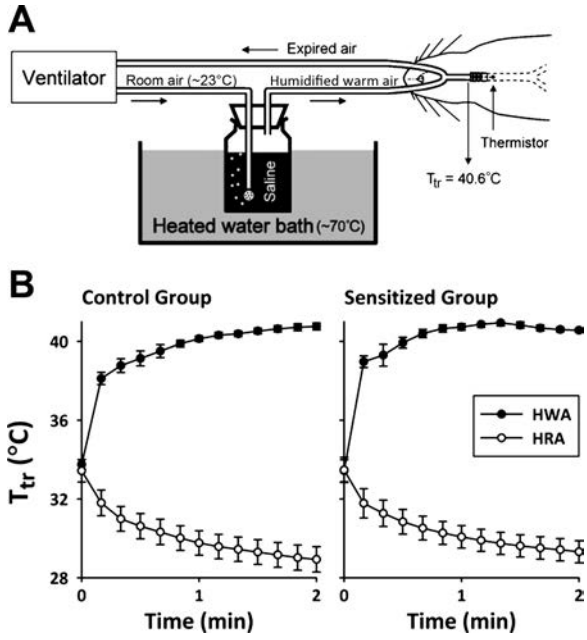

Fig. 1. *A*: schematic drawing of the experimental setup for delivery of humidified warm air (HWA) into the lung of anesthetized Brown-Norway rats. T_{tr}, air temperature in trachea. *B*: change in T_{tr} during the 2-min hyperventilation with HWA and humidified room air (HRA; water bath was kept at room temperature ~23°C) in control (*left*) and ovalbumin (Ova)-sensitized (*right*) rats. Data are means ± SE for 6 rats.

Experimental Protocols

Eight series of experiments were carried out in this study.

Series 1. To determine whether HWA hyperventilation induced bronchoconstriction in Ova-sensitized rats and, if so, whether the effect was generated by the increase in T_{tr}, in each rat R_L and C_{dyn} were measured continuously on a breath-by-breath basis for 1 min before (as the baseline) and for 15 min immediately after the hyperventilation. The responses to hyperventilation with HWA were then compared between control and Ova-sensitized rats. To determine the effect of HWA, the responses to hyperventilation with HWA and HRA were then compared in the same rat; the sequence of HWA and HRA challenges was altered between animals to achieve a balanced design, and 60–90 min elapsed between two challenges for recovery.

Series 2. To determine whether the response induced by HWA in Ova-sensitized rats was temperature dependent, animals were challenged with humidified air of three different temperatures that resulted in three different levels of end-tidal T_{tr}, high (40.6°C), intermediate (39.6°C), and room air (29.4°C), and 60–90 min was allowed to elapse between two challenges.

Series 3. To determine whether the HWA-induced responses in Ova-sensitized rats were reproducible, the same HWA challenge was repeated after 60–90 min in the same rat.

Series 4. Responses to HWA challenges were compared in the Ova-sensitized rat when the humidity in HWA was generated from isotonic saline and distilled water.

Series 5. To evaluate the influence of humidity alone in the HWA-induced responses, responses to hyperventilation with HWA and saline aerosol were compared in the same Ova-sensitized rat; the same amount of water content (~80 mg/l of air) was generated by aerosol (Aeroneb Pro; Aerogen Nektar, Galway, Ireland) and delivered in the identical manner as that in the HWA challenge, except that the saline aerosol was administered at room temperature (~23°C).

Series 6. To evaluate the role of cholinergic reflex in the HWA-evoked change in R_L, responses to HWA in Ova-sensitized rats were compared between before and after pretreatment with atropine sulfate (0.1 mg/kg iv), a muscarinic receptor antagonist.

Series 7. To test whether the effect of HWA was caused by smooth muscle contraction, responses to HWA in Ova-sensitized rats were compared between before and after pretreatment with formoterol (10 μg/kg iv), a selective β_2 agonist.

Series 8. To investigate a possible involvement of the endogenous tachykinins, the HWA-induced responses in Ova-sensitized rats were compared between before and after pretreatment with a combination of neurokinin (NK) receptor type 1 and 2 (NK-1 and NK-2) antagonists L-732138 (6 mg/kg iv) and SR-48968 (0.3–1 mg/kg iv), respectively. The same doses of SR-48968 and L-732138 almost completely prevented the increase in R_L evoked by bolus intravenous injection of neurokinin A (NKA; 0.2 μM, 0.15 ml) and substance P (SP; 0.2–0.5 μM, 0.15 ml) in anesthetized rats: the peak ΔR_L values (above the baseline) after NKA injection were 137 ± 31% before and 24 ± 4% ($P < 0.01$, $n = 5$) after the pretreatment with SR-48968 and L-732138, and the peak ΔR_L values after SP injection were 29 ± 3% before and 16 ± 2% ($P < 0.05$, $n = 5$) after the pretreatment with SR-48968 and L-732138. These results are in agreement with the effective blocking doses of these NK receptor antagonists reported in the literature (25, 45).

Statistical Analysis

In each experiment, baseline R_L and C_{dyn} were averaged over 1 min (i.e., 60 breaths) before HWA or HRA challenge; the peak responses were averaged over 60 consecutive breaths within the first 3 min after HWA or HRA challenge. Data were compared with a paired *t*-test or a two-way repeated-measures analysis of variance (ANOVA). When the ANOVA showed a significant interaction, pairwise comparisons were made with a post hoc analysis (Fisher's least significant difference). *P* values of <0.05 were considered significant. Data are reported as means ± SE.

Materials

Solution of Ova (Sigma-Aldrich) was prepared daily at a concentration of 1.25% (wt/vol) in saline. Imject Alum as adjuvant was purchased from Pierce Biotechnology (Rockford, IL). Stock solutions of chemical agents were prepared as follows: L-732138 (Tocris, Ellisville, MO) was dissolved in DMSO (Sigma-Aldrich) at a concentration of 25 mg/ml; SR-48968 (Sanofi Recherche, Montpellier, France) in polyethylene glycol (average mol wt 200; Sigma-Aldrich) at a concentration of 10 mg/ml; formoterol (Sigma-Aldrich) in DMSO at a concentration of 1 mg/ml; and atropine sulfate (Sigma-Aldrich) in isotonic saline at a concentration of 10 mg/ml. Solutions of these chemical agents at the desired concentrations for injections were then prepared daily by dilution with saline based on the animal's body weight.

RESULTS

This study was carried out in a total of 41 Brown-Norway rats with an average body weight of 270 ± 5 g. Some of the animals were used in more than one series of experiments. Hyperventilation with HWA led to an increase in peak T_{tr} from 33.4 ± 0.6°C to 40.6 ± 0.1°C ($n = 6$; $P < 0.001$) in the sensitized group and from 33.8 ± 0.2°C to 40.8 ± 0.2°C ($n = 6$; $P < 0.001$) in the control group (Fig. 1*B*). In contrast, hyperventilation with HRA caused a slight decrease in T_{tr} (from 33.5 ± 0.6°C to 29.3 ± 0.6°C in sensitized group and from 33.4 ± 0.6°C to 28.9 ± 0.6°C in control group). In seven rats, the baseline arterial pH, P_{O_2}, and P_{CO_2} were 7.50 ± 0.03, 95.3 ± 4.3 mmHg, and 36.8 ± 1.7 mmHg, respectively, and they did not change when measured during the last 30 s of hyperventilation with HWA in the same animals: arterial pH, P_{O_2}, and P_{CO_2} were 7.51 ± 0.02 ($P > 0.5$), 98.4 ± 3.8 mmHg ($P > 0.5$), and 34.5 ± 1.0 mmHg ($P > 0.05$), respectively.

Hyperventilation with HWA induced an increase in R_L in Ova-sensitized rats but not in control rats. The response occurred immediately after the HWA challenge and lasted for >10 min (Figs. 2 and 3). R_L increased from 0.128 ± 0.004 $cmH_2O \cdot ml^{-1} \cdot s$ (60-breath average) before to 0.212 ± 0.013 $cmH_2O \cdot ml^{-1} \cdot s$ (60-breath average) after HWA ($P < 0.01$, $n = 6$; Fig. 3). In sharp contrast, the same HWA challenge did not generate any detectable changes in R_L in control rats (from 0.124 ± 0.007 to 0.133 ± 0.008 $cmH_2O \cdot ml^{-1} \cdot s$, $P > 0.05$, $n = 6$; Fig. 3). Hyperventilation with HRA did not cause any significant change in R_L in either control or sensitized rats (Figs. 2 and 3). Furthermore, there was no significant change in C_{dyn} after either HWA or HRA challenge in either sensitized or control rats (Figs. 2 and 3).

The increase in R_L was temperature dependent; a slightly smaller increase in T_{tr} (39.6 ± 0.2°C) generated a smaller but significant and consistent increase in R_L (from 0.119 ± 0.005 to 0.184 ± 0.017 $cmH_2O \cdot ml^{-1} \cdot s$) in sensitized rats (Fig. 4).

The increases in R_L were reproducible when the same HWA challenge was repeated after 60–90 min in the same rats (Fig. 5*A*); there were no significant differences between the responses to the first and second HWA challenges ($P > 0.4$, $n = 5$).

The responses to HWA generated from distilled water and isotonic saline were compared in the same Ova-sensitized rats; the sequences of these two tests were alternated between animals. There were no significant differences between the responses of R_L to HWA generated from distilled water and isotonic saline ($P > 0.5$, $n = 6$; Fig. 5*B*).

The increase in R_L was not caused by the humidity in the HWA alone because the same water content as in HWA delivered by saline aerosol for 2 min did not generate any increase in R_L (Fig. 6), while the aerosol inhalation caused a small decrease in T_{tr} (from a baseline of 32.9 ± 0.6°C to 29.0 ± 0.4°C after challenge).

Pretreatment with atropine sulfate did not reduce the HWA-induced increase in R_L (Fig. 7*A*); the increases in R_L after HWA challenge were 0.095 ± 0.023 and 0.078 ± 0.011 $cmH_2O \cdot ml^{-1} \cdot s$ before and after atropine, respectively, in Ova-sensitized rats ($P > 0.5$, $n = 5$). In a separate study, we verified that pretreatment with the same dose of atropine (0.1 mg/kg iv) completely prevented a pronounced increase in R_L evoked by inhalation of aerosolized acetylcholine chloride (200 mM; 5 μl delivered by respirator in 3 or 4 consecutive tidal breaths) in anesthetized rats prepared in the identical manner; before atropine ACh inhalation increased R_L from 0.09 ± 0.01 to 0.40 ± 0.11 $cmH_2O \cdot ml^{-1} \cdot s$ ($P < 0.001$, $n = 4$), and after atropine the same ACh challenge did not change R_L (from 0.09 ± 0.01 to 0.10 ± 0.01 $cmH_2O \cdot ml^{-1} \cdot s$; $P > 0.05$, $n = 4$).

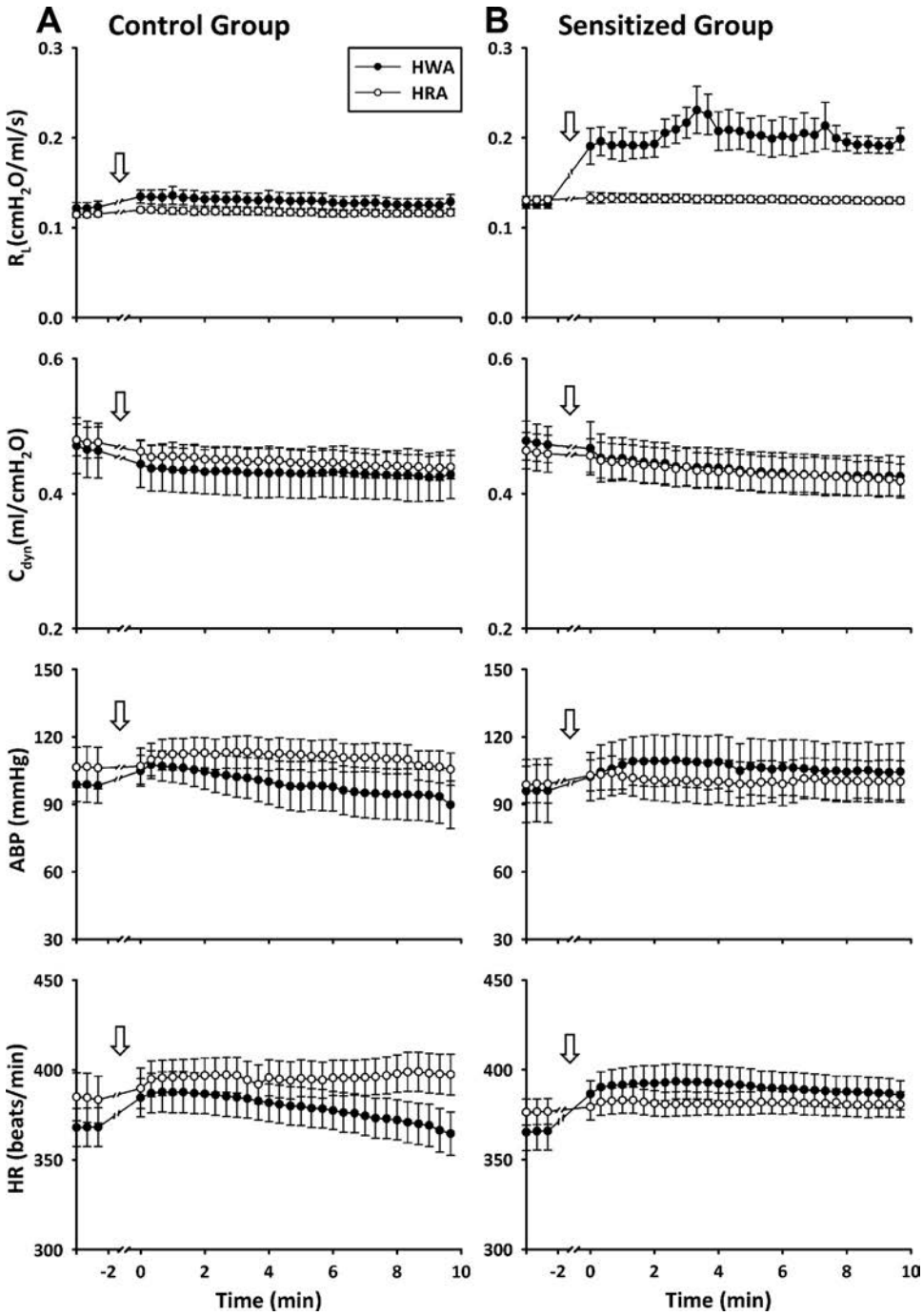

Fig. 2. Effect of hyperventilation with HWA and HRA on total pulmonary resistance (R_L), dynamic lung compliance (C_{dyn}), arterial blood pressure (ABP), and heart rate (HR) in control (*A*) and Ova-sensitized (*B*) rats. Responses were not recorded during hyperventilation (arrows), which was administered between −2 and 0 min. Data before time −2 min represent baseline values; 60–90 min was allowed to elapse between 2 challenges. Each data point was averaged over 20 consecutive breaths. Data are means ± SE for 6 rats.

Pretreatment with formoterol completely abolished the increase in R_L caused by the HWA challenge in sensitized rats (Fig. 7*B*); the peak increases in R_L were 0.092 ± 0.024 and 0.016 ± 0.011 $cmH_2O \cdot ml^{-1} \cdot s$ before and after formoterol, respectively ($P < 0.05$, $n = 6$).

Pretreatments with L-732138 and SR-48968 also completely abolished the increase in R_L caused by the HWA challenge in sensitized rats (Fig. 7*C*); the immediate increases in R_L after the HWA challenge were 0.090 ± 0.014 and 0.023 ± 0.007 $cmH_2O \cdot ml^{-1} \cdot s$ before and after the combined treatments with L-732138 and SR-48968, respectively ($P < 0.01$, $n = 6$). A slight increase in R_L ($P < 0.05$, $n = 6$) was detected at 10 min after the HWA challenge (Fig. 7*C*).

DISCUSSION

This study showed that an increase in T_{tr} to 40.6°C after hyperventilation with HWA for 2 min triggered a significant and sustained increase in airway resistance in Ova-sensitized rats. This bronchoconstrictive effect of HWA was reversible and reproducible in the same rats. In sharp contrast, the same HWA hyperventilation challenge did not induce any bronchoconstriction in control rats, despite the same increase in T_{tr}. The intensity of the HWA-induced bronchoconstriction was temperature dependent; a slightly smaller increase in T_{tr} (39.6°C) caused a smaller increase in airway resistance in Ova-sensitized rats. This effect was not caused by humidity

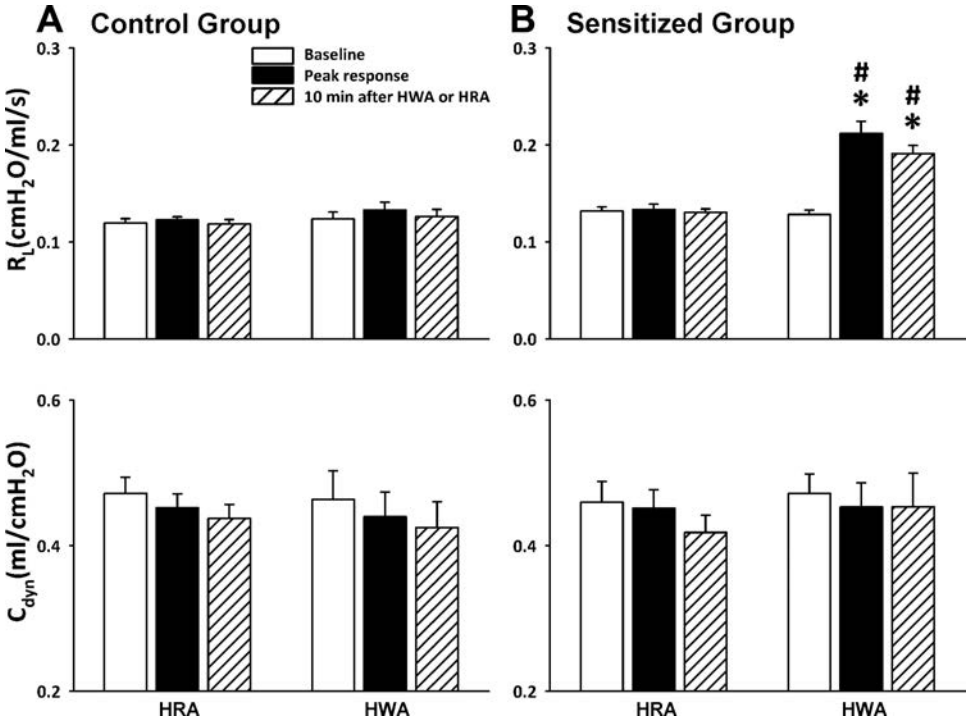

Fig. 3. Comparison of the responses of R_L and C_{dyn} to hyperventilation with HWA and HRA in control (*A*) and Ova-sensitized (*B*) rats. Open bars, baseline data averaged over 1 min before; filled bars, peak responses averaged over 1 min after; hatched bars, responses 10 min after HWA or HRA challenge; 60–90 min was allowed to elapse between 2 challenges. Data are means ± SE for 6 rats. *Significantly different from baseline ($P < 0.05$); #significant difference when corresponding data between HWA and HRA were compared ($P < 0.05$).

alone, because the same water content delivered by saline aerosol at room temperature failed to generate any significant bronchoconstriction. Also, the same effect was observed when the humidity was generated from distilled water. These results illustrated that chronic airway inflammation caused by allergen sensitization rendered the airways more vulnerable to airway hyperthermia and induced pronounced bronchoconstriction. This HWA-evoked bronchoconstriction was prevented by pretreatment with a combination of both NK-1 and NK-2 antagonists, indicating that an endogenous release of tachykinins was primarily responsible.

There are three primary types of mammalian tachykinins: SP, NKA, and neurokinin B (NKB). In the respiratory tract, SP and NKA (both derived from the same precursor gene preprotachykinin A) are mainly synthesized in the cell body of nonmyelinated (C fiber) sensory nerves and transported along the axon toward the peripheral terminals, where they are stored in large granular vesicles and released from the sensory endings upon a surge of calcium influx triggered by membrane depolarization. SP and NKA are frequently colocalized and coreleased from the same pulmonary sensory neurons. The actions of these tachykinins are mediated through three distinct types of receptors, NK-1, NK-2, and NK-3, that are expressed in the plasma membrane of various effector cells in the airways and have preferential affinities for the three major types of endogenous tachykinins, SP, NKA, and NKB, respectively (21, 29). Activation of NK-1 receptors by SP causes an increase in vascular permeability to plasma macromolecules, adhesion of leukocytes to the vascular endothelium, and vascular smooth muscle relaxation in the arterioles; these effects together induce vasodilatation in the microvessels and mucosal edema in the airways (21, 31, 33). In addition, the NK-1 receptor is known to be involved in initiating the chemotactic reaction and leukocyte infiltration into the airway and the consequent release of inflammatory mediators and cytokines (31, 33). Indeed, the diverse immunomodulatory effects of tachykinins, mainly mediated through activation of NK-1 receptors, on mast cells, neutrophils, lymphocytes, and macrophages in the respiratory tract have been extensively documented (21). The NK-2 receptor is expressed primarily on the airway smooth muscle, and its activation induces bronchoconstriction (21, 28, 41). The NK-2 receptor is also found on the airway cholinergic ganglion neurons and postganglionic prejunctional cholinergic nerve terminals, which upon activation can facilitate ganglionic transmission and acetylcholine release (28, 41). NKB is rarely found in the lung and airways (20), but the NK-3 receptor, present on the cholinergic ganglion neurons, can be also activated by SP and NKA and facilitate the synaptic transmission (8). Combined effects of these neuropeptides are believed

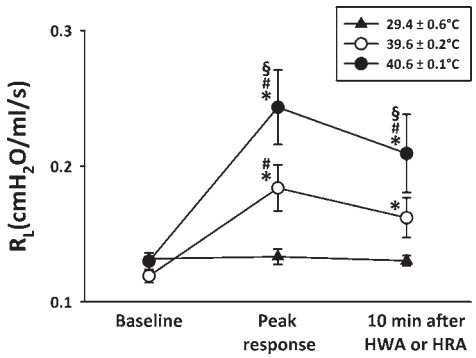

Fig. 4. Temperature-dependent effect of HWA challenge on R_L in Ova-sensitized rats. Three different levels of T_{tr} were maintained during the test: high (40.6°C), intermediate (39.6°C), and room air (29.4°C); 60–90 min was allowed to elapse between 2 challenges. Data are means ± SE for 6 rats. *Significantly different from baseline ($P < 0.05$); #significantly different from response to room air temperature ($P < 0.05$); §significantly different from response to intermediate temperature ($P < 0.05$).

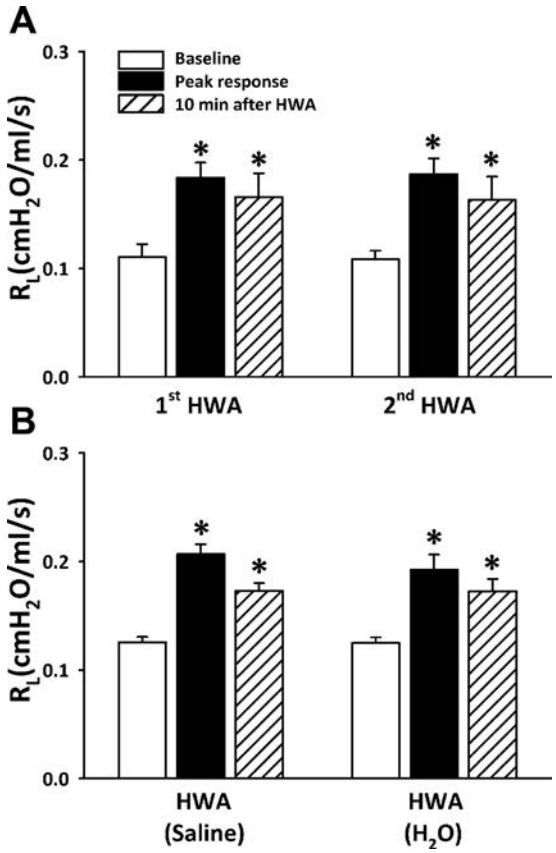

Fig. 5. *A*: reproducibility of the responses of RL to 2 consecutive HWA challenges in the same group of Ova-sensitized rats. *B*: responses of RL to HWA were compared in the same group of Ova-sensitized rats when humidity was generated from distilled water and isotonic saline. Sixty to ninety minutes were allowed to elapse between 2 challenges. Data are means ± SE; $n = 5$ in *A* and $n = 6$ in *B*. *Significantly different from baseline ($P < 0.05$).

to contribute to the local "axon reflexes" that are generated by stimulation of C-fiber afferents but cannot be abolished by bilateral vagotomy or atropine (21, 28, 41). Sustained and/or intense stimulation of C-fiber afferents can lead to the development of "neurogenic inflammatory reaction" in the airways (2, 21, 32, 41).

The species-dependent variation in the functions of these different NK receptors is well documented; for example, SP induces airway smooth muscle contraction in guinea pig and hamster airways via the activation of both the NK-2 and, to a lesser extent, the NK-1 receptors (21, 28). Thus capsaicin administered by injection or inhalation evokes bronchoconstriction in these species via both the centrally mediated cholinergic reflex pathway and the local axon reflex responses. However, stimulation of vagal bronchopulmonary C-fiber afferents by capsaicin does not generate a conspicuous and consistent bronchoconstriction in rats (43, 44); the lack of response may be related to the fact that SP released from C-fiber endings upon stimulation can activate NK receptors in the epithelial and endothelial cells and trigger the secondary release of bronchodilating mediators such as prostaglandin E_2 and nitric oxide from these cells in rat airways (11, 25, 44), which can mask the centrally mediated reflex bronchoconstriction.

Chronic allergic inflammation caused by active sensitization is known to increase the expression of preprotachykinin mRNA in the vagal sensory neurons and the amount of tachykinin synthesis and release in the airways (15). In addition, allergic airway inflammation is known to inhibit the enzyme activity of neutral endopeptidase (NEP) that is present on the membranes of various cell types (including epithelium and nerve fibers) in the airways (9). NEP can cleave tachykinins immediately after their release (19), and therefore the attenuated NEP activity may also contribute to the enhanced bronchoconstrictive effect of endogenous tachykinins. Chronic airway inflammation induced by Ova sensitization can also cause a phenotypic switch of both the tachykinergic innervation and the expression of transient receptor potential vanilloid type 1 receptors (TRPV1) in the airways (34, 46). Under normal conditions, expressions of both tachykinins and TRPV1 are found almost exclusively in capsaicin-sensitive, neurofilament-negative C-fiber afferents innervating the guinea pig airways. However, in Ova-sensitized guinea pigs SP was found in ~30% of the large-diameter, neurofilament-positive neurons (34). A recent study further demonstrated that chronic allergic inflammation in Brown-Norway rats actively sensitized with Ova induced a significant increase in the expression of TRPV1 in bronchopulmonary neurons in nodose ganglia, mainly in neurofilament-positive (myelinated) neurons (46). Indeed, capsaicin sensitivity was detected in some of the vagal myelinated (A fiber) afferents that normally do not exhibit capsaicin sensitivity (46), and the baseline activity and sensitivities of

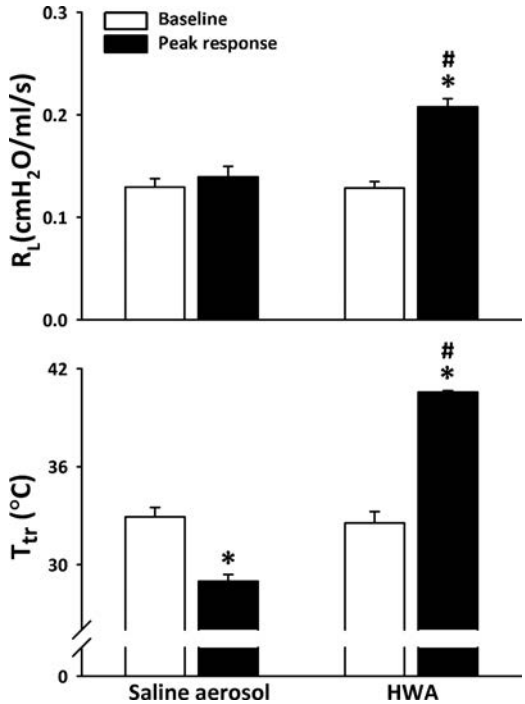

Fig. 6. Comparison of responses of RL (*top*) and T_{tr} (*bottom*) to hyperventilation with HWA and with saline aerosol in the Ova-sensitized rats. The same amount of water content (77.6 ± 5.9 mg/l of air) was delivered by aerosol and by HWA in the same manner in the same rats, but the saline aerosol was generated by a nebulizer and delivered at room temperature (~23°C); 60–90 min was allowed to elapse between 2 challenges. Data are means ± SE for 6 rats. *Significantly different from baseline ($P < 0.05$); #significant difference when corresponding data between HWA and saline aerosol were compared ($P < 0.05$).

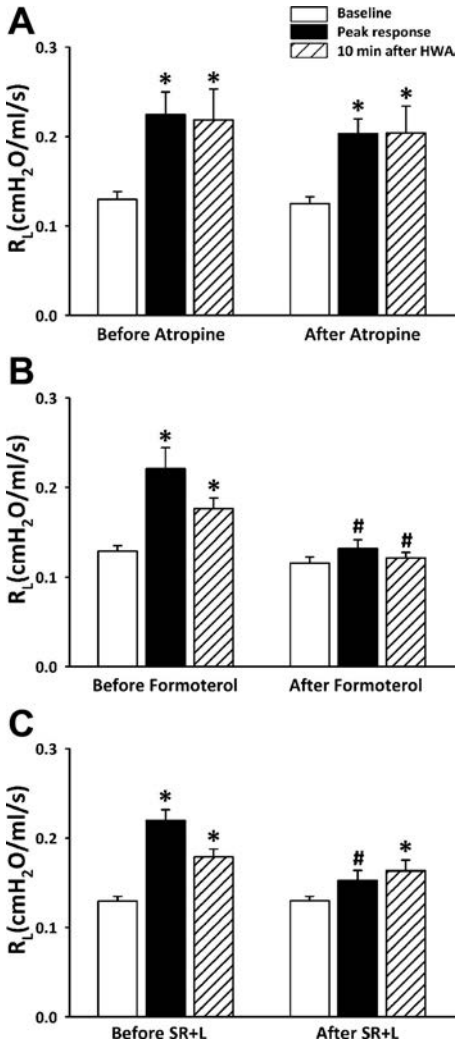

Fig. 7. *A*: comparison of the responses of R_L to hyperventilation with HWA before and after pretreatment with atropine sulfate (0.1 mg/kg iv). *B*: comparison between before and after pretreatment with formoterol (10 μg/kg iv). *C*: comparison between before and after pretreatment with a combination of L-732138 (L; 6 mg/kg iv) and SR-48968 (SR; 0.3–1.0 mg/kg iv). Sixty to ninety minutes was allowed to elapse between 2 challenges. Data are means ± SE for 6 rats. *Significantly different from baseline ($P < 0.05$); #significant difference when corresponding data between before and after pretreatment were compared ($P < 0.05$).

pulmonary C fibers to both chemical stimulants and lung inflation were also markedly elevated in Ova-sensitized rats (47). The upregulation of both tachykinin and TRPV1 expression in the airway sensory nerves of Ova-sensitized animals may have contributed, to some extent, to the increase in airway resistance after the HWA hyperventilation challenge observed in this study.

Previous investigators have reported the expression of TRPV channels (TRPV2 and TRPV1) in mast cells (4, 42). Thus an increase in airway temperature generated by the HWA challenge may activate TRPVs and trigger mast cell degranulation and release of histamine and other bronchoactive autacoids, which may have contributed to the bronchoconstriction. In addition, previous studies have extensively documented the expression of NK-1 receptors in mast cells and other inflammatory cells (21). Thus in either direct activation of TRPVs by high temperature or indirect activation by SP released from bronchopulmonary C fibers, a possible involvement of mast cell degranulation should be considered in the airway response to HWA. In particular, such an action mediated through mast cells is expected to be enhanced in Ova-sensitized rats. Our observation that HWA-induced bronchoconstriction was completely prevented by pretreatment with NK receptor antagonists seems to support the latter (i.e., involvement of the NK-1 receptor instead of TRPVs). We should also point out that the elevated airway temperature during the HWA challenge in this study was substantially lower than the activation threshold for TRPV2 (>50°C) (3).

The HWA-evoked increase in airway resistance in Ova-sensitized rats was completely prevented by pretreatment with formoterol, a bronchodilator, which seems to suggest that the bronchoconstrictive effect of HWA involved contraction of airway smooth muscles. However, we cannot rule out the possibility that the increase in airway resistance may have also resulted, partially or totally, from the protein extravasation and mucosa edema in the airway walls of sensitized animals. Previous investigators have demonstrated that β_2 agonists, such as formoterol, can attenuate the plasma extravasation by reducing the number of endothelial gaps in postcapillary venules, which is probably mediated through an increase in intracellular cAMP resulting from β_2-receptor activation in the endothelial cells (1). Furthermore, pretreatment with β_2 agonist can also reduce the number of eosinophils and neutrophils adhered to the vascular endothelium and thus diminish the inflammatory reaction and the resulting bronchoconstriction (6).

The above assessments of our results suggest that HWA challenge elevated the activity of vagal bronchopulmonary C-fiber afferents in Ova-sensitized rats. Indeed, results obtained from a parallel study carried out in our lab support this hypothesis. Using the same preparations and animal model, we recorded "single-fiber" activities of vagal bronchopulmonary C-fiber afferents before and after HWA hyperventilation challenge and compared them between control and Ova-sensitized rats (27). The study showed that an increase in T_{tr} by HWA challenge similar to that in this study markedly elevated the baseline activity of these afferents and further amplified their hypersensitivity to chemical stimulation in Ova-sensitized rats but not in control rats (27).

The mechanism(s) involved in the HWA-induced C-fiber hypersensitivity is not fully understood, but an increase in the temperature sensitivity of these C-fiber afferents is probably involved. The primary sensors for detecting warm and hot temperature in mammalian species are TRPV channels. TRPVs are a family of ion channels containing six transmembrane domains that form nonselective, non-voltage-gated cationic channels (36). Each of the four subtypes of TRPVs (TRPV1–4) is activated in a different temperature range (3, 12). In light of our earlier electrophysiological study in isolated rat vagal pulmonary sensory neurons (35), the increase in T_{tr} generated by HWA challenge in the present study is likely to activate more than one type of these TRPV channels. Among them, TRPV1, generally considered as a reliable biomarker for the C-fiber sensory nerves in rat lung (18, 23), accounts for approximately half of the total current evoked by a similar increase in temperature in isolated pulmonary sensory neurons (35).

A recent study conducted in our laboratory reported that hyperventilation with HWA triggered an immediate and re-

versible bronchoconstriction in patients with mild and stable asthma but not in healthy subjects (17). The degree and pattern of bronchoconstriction were similar to those observed in the Ova-sensitized rats in the present study. Accompanying the bronchoconstriction, breathing HWA also triggered coughs in these patients, suggesting an involvement of activation of airway sensory nerves (17). Although we hypothesized that the HWA-induced increase in T_{tr} activated bronchopulmonary C fibers, direct evidence could not be obtained in these patients. The bronchoconstriction in these patients was completely prevented by pretreatment with ipratropium, indicating a dominant role of cholinergic reflex, which is a sharp contrast to our finding in this study that pretreatment with atropine in the Ova-sensitized rats did not significantly diminish the HWA-induced response (Fig. 7*A*). Several factors are probably involved in causing this clear discrepancy. A major factor to be considered is the well-documented variance between different species (especially between large mammals and rodents) in the relative contributions of centrally mediated cholinergic and local tachykininergic mechanisms to the airway responses to C-fiber stimulation (8, 14, 21, 22), as described previously. In addition, in our earlier study of patients with asthma the airway responses to HWA were measured in awake subjects, whereas in the present study rats were anesthetized during the HWA challenge; it is well recognized that reflex responses mediated through the central nervous system are generally suppressed during anesthesia. Another contributing factor may be related to the differences in the nature, stage, and severity of the pathological features between this animal model of allergic asthma and the chronic disease of human asthma.

In conclusion, an increase in airway temperature within the physiological range evoked an immediate and sustained increase in airway resistance in Ova-sensitized rats but not in control rats. Tachykinins released from the vagal bronchopulmonary C-fiber endings in response to airway hyperthermia appear to be primarily responsible for generating this bronchoconstrictive effect.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.-C.H. and R.-L.L. performed experiments; C.-C.H. and R.-L.L. analyzed data; C.-C.H., R.-L.L., and Y.S.L. prepared figures; C.-C.H., Y.S.L., and L.-Y.L. drafted manuscript; Y.S.L. and L.-Y.L. interpreted results of experiments; Y.S.L. and L.-Y.L. edited and revised manuscript; L.-Y.L. conception and design of research; L.-Y.L. approved final version of manuscript.

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Acid-sensing by airway afferent nerves

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ABSTRACT

Inhalation of acid aerosol or aspiration of acid solution evokes a stimulatory effect on airway C-fiber and Aδ afferents, which in turn causes airway irritation and triggers an array of defense reflex responses (e.g., cough, reflex bronchoconstriction, etc.). Tissue acidosis can also occur locally in the respiratory tract as a result of ischemia or inflammation, such as in the airways of asthmatic patients during exacerbation. The action of proton on the airway sensory neurons is generated by activation of two different current species: a transient (rapidly activating and inactivating) current mediated through the acid-sensing ion channels, and a slowly activating and sustained current mediated through the transient receptor potential vanilloid type 1 (TRPV1) receptor. In view of the recent findings that the expression and/or sensitivity of TRPV1 are up-regulated in the airway sensory nerves during chronic inflammatory reaction, the proton-evoked irritant effects on these nerves may play an important part in the manifestation of various symptoms associated with airway inflammatory diseases.

1. Introduction

The concentration of hydrogen ion in body fluids can be elevated substantially during various physiological and pathophysiological conditions. For example, lactic acid is produced in large quantities by the skeletal muscles during anaerobic exercise in healthy individuals [1]. Because lungs are perfused by the total venous return, they are fully exposed to the lactic acid produced by the peripheral tissues. Furthermore, the production of lactic acid is known to be elevated locally in the inflamed and/or ischemic tissues [2,3]. Indeed, in patients during asthmatic attack, the pH of the airway vapor condensate of exhaled gas is reduced to 5.23, as compared to 7.65 in healthy individuals [4,5]. This abnormally low airway pH returns to normal after anti-inflammatory therapy, suggesting the tissue inflammation as the origin of airway acidosis [4]. In addition, acidosis resulting from retention of carbon dioxide in the body fluid is one of the most common and debilitating symptoms in patients with severe chronic pulmonary diseases. All these findings indicate that tissue acidosis occurs in the airways and lungs in health as well as in disease conditions. Although the airway responses evoked by an increase in acidity in the respiratory tract has been extensively documented, recent studies began to further identify the specific types of ion channels involved and uncover the underlying mechanisms of the airway irritation caused by tissue acidification.

2. Airway irritation evoked by acid

Inhalation or aspiration of acid solution causes airway irritation and triggers various airway defense reflex responses such as cough and bronchoconstriction. More than four decades ago, Simonsson et al. first reported that inhalation of citric acid aerosol (20% solution) evoked an abrupt increase in airway resistance in patients with asthma, and the response was completely abolished by a pretreatment with atropine [6] (Fig. 1A). Accompanying the immediate bronchoconstriction, citric acid aerosol inhalation challenge also evoked airway irritation and vigorous coughs in these patients, suggesting that cholinergic reflex elicited by acid stimulation of airway sensory nerves was responsible [6]. Similarly, the bronchoconstriction induced by right heart injection of acid solution was mostly abolished by atropine in anesthetized newborn dogs [7]. However, in anesthetized guinea pigs, the bronchoconstrictive response to inhaled citric acid aerosol was mediated in a large part through the action of sensory neuropeptides such as tachykinins and calcitonin gene-related peptide (CGRP) released from these sensory endings upon activation because the airway responses were blocked by pretreatment with specific antagonists of neurokinin receptors [8]. The evidence of a dominant role of tachykinins in regulating the airway responses to inhaled irritants such as acid is well documented in rodents [9], but their relative

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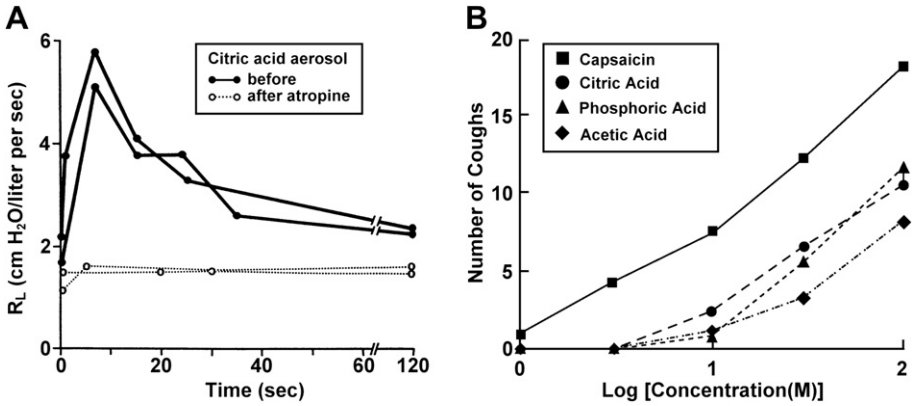

Fig. 1. Airway irritation generated by inhaled acid aerosol in humans. *Panel A*, changes in total lung resistance (RL) after inhalation of one breath of citric acid aerosol (20% solution) before (solid lines) and 10 min after injection of atropine sulfate (2 mg, intravenous; dotted lines) in a patient with asthma; the inhalation challenge was repeated in the same patient to test the reproducibility. *Panel B*, mean dose responses of cough to inhalation challenges of aerosolized capsaicin ($n = 18$), citric acid ($n = 48$), phosphoric acid ($n = 22$) and acetic acid ($n = 26$) in healthy subjects (age 18–60 years; each subject was tested in at least two study series). (adapted from Ref. [6,21]).

contribution (as compared to the cholinergic reflex) in the airway responses to these irritants remains to be clearly defined in humans.

In patients with gastroesophageal and laryngopharyngeal reflux diseases, aspiration of gastric acid is known to trigger reflex bronchoconstriction and cough [10]. It has been clearly demonstrated in experimental animals that these reflex responses were elicited by acid stimulation of the sensory nerves innervating larynx and trachea [11–13]. In addition, gastric acid can also stimulate the vagal afferents innervating the distal segment of the esophagus, which may be involved in eliciting the reflex bronchoconstriction triggered by gastroesophageal reflux in asthmatics [10,14].

Association of inhaled acid and asthma symptoms is also frequently reported when asthmatics are exposed to acid fog or aerosol in the environmental air [15–17], and the airway irritation, bronchoconstriction and coughing are also attributed to acid stimulation of airway sensory nerves [18]. These symptoms can be further aggravated during exercise because air (and acid) intake is increased during hyperventilation and the filtering function of the nasal passage is bypassed when breathing via the mouth.

Inhaled citric acid aerosol was first used for experimental production of cough in man about six decades ago [19], and remains as one of the most frequently used agent for testing cough responsiveness in humans [20–23]. The cough response to inhaled citric acid increased in a dose-dependent pattern in healthy subjects (Fig. 1B). Other forms of acid solution (e.g., acetic acid, phosphoric acid, etc.) in the same range of pH (approximately 1.5–2.5) were similarly effective in generating cough, indicating a key role of hydrogen ion in the stimulatory effect on airway sensory nerves [20,21] (Fig. 1B). The cough sensitivity to citric acid was heightened in patients with airway inflammatory diseases such as COPD [24,25].

3. Airway sensory nerves stimulated by acid

It is well documented that increasing acidity in the extracellular fluid activated nociceptive nerve endings in various somatic and visceral tissues, and evoked pain sensation [26–29]. In the respiratory tract, indirect evidence of an activation of C-fiber sensory nerves by proton was first reported in an isolated perfused guinea-pig lung preparation. Constant perfusion of pulmonary arteries with the acid buffer at pH of 5.0 caused the release of tachykinins and CGRP from these sensory nerves, which could be blocked by capsazepine, a selective antagonist of the "capsaicin receptor", suggesting that "capsaicin receptors" were activated during acid stimulation [30,31]. Direct evidence of acid stimulation of C-fiber sensory nerves was established by Fox and coworkers in an isolated airway-nerve preparation [32]. They demonstrated that C-fiber afferents innervating the guinea pig trachea, but not Aδ fibers, were stimulated when the pH of the perfusing buffer was reduced to 5.0. Their study further showed that the stimulatory effect of proton is mediated through activation of the capsaicin receptor because it could be abolished by capsazepine [32].

In anesthetized rats, lactic acid injected as a bolus into the right atrium caused a transient decrease in pulmonary venous blood pH (dropped from 7.41 to 7.09–7.29), and a short but intense burst of afferent activities in pulmonary C-fibers [33] (e.g., Fig. 2). This stimulatory effect of lactic acid was dose dependent. Formic acid, with a pK_a value (the negative logarithm of the acid dissociation constant) similar to that of lactic acid (3.79) and thus at the same molar concentration decreasing blood pH to the same degree, evoked a similar response in pulmonary C-fibers, further suggesting that hydrogen ions were primarily responsible for the action [33]. The stimulatory effect of lactic acid was abrogated by capsazepine in 75% (8 out of 12) of the pulmonary C-fibers tested but was not altered in the remaining 25% (Fig. 2), despite that this dose of capsazepine was sufficient to block the stimulatory effect of a large dose of capsaicin (five folds of its threshold dose). This finding suggested that an activation mechanism not mediated through the capsaicin receptor was also involved in the action of hydrogen ion on some of pulmonary C-fiber endings. In addition, right atrial injection of lactic acid also stimulated a small sub-population of the rapidly adapting receptors (RARs) in the rat lung [34] (Fig. 3A), which was somewhat surprising because RARs are recognized as mechanosensitive airway afferents, and generally do not exhibit chemosensitivity [34].

It has been reported that acid solution can act on the airway tissue surrounding the sensory terminals or circulating blood, and trigger the release of certain chemical mediators such as thromboxanes and prostaglandins [31,35], which may in turn cause a secondary stimulatory and/or sensitizing effect on these nerve endings [31,32,36]. However, a possible involvement of cyclooxygenase metabolites in the stimulatory effect of lactic acid can be ruled out because the responses were not affected by pretreatment with indomethacin [37].

In a more recent study, Kollarik and Undem recorded separately from vagal nodose and jugular ganglia in an isolated airway-nerve preparation similar to that used by Fox et al. [32], and found that

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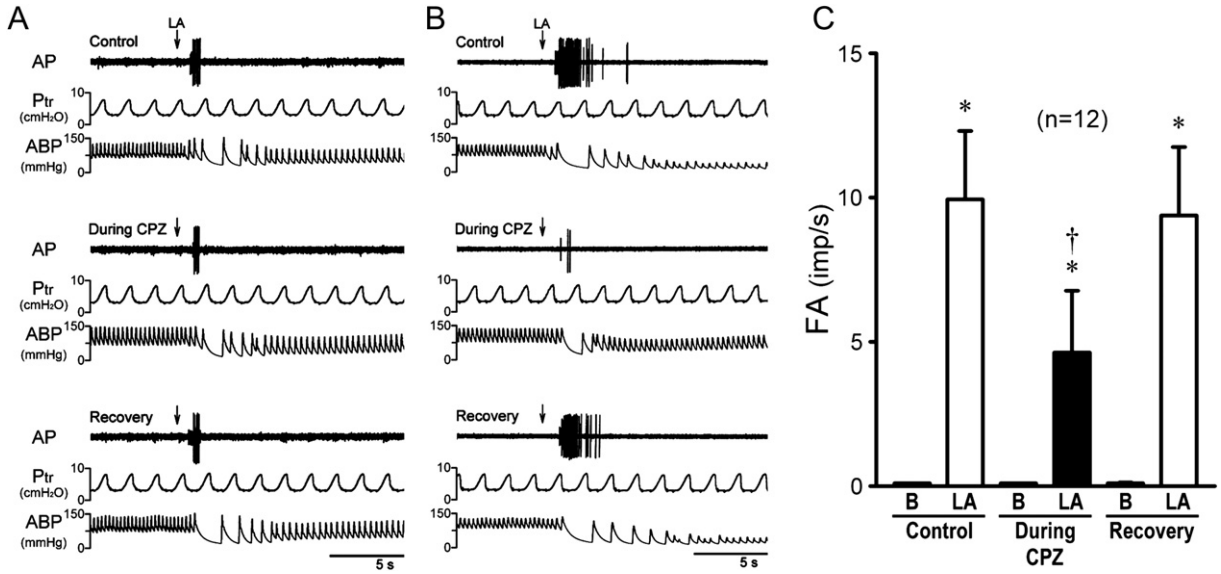

Fig. 2. Effect of capsazepine (CPZ), a selective TRPV1 antagonist, on pulmonary C-fiber responses to lactic acid (LA) in rats. *Panel A*, LA (0.2 mmol/kg; injectate volume = 0.2 ml; pH = 2.1) was injected as a bolus into the right atrium at the arrow during control (top), constant intravenous infusion of CPZ (0.3 mg/kg/min for 5 min; middle) and recovery (20 min after termination of CPZ; bottom) in an anesthetized, open-chest and mechanically ventilated rat (weight 348 g). This dose of CPZ administered was sufficient to block the stimulatory effect of a large dose of capsaicin (2.0 μg/kg; ~5 folds of its threshold dose; data not shown). Note that two C-fibers distinguished by different spike heights were recorded; locations of receptors: one in upper and one in middle lobe of the right lung. AP, action potentials; Ptr, tracheal pressure; ABP, arterial blood pressure. *Panel B*, experimental procedures and doses identical to that in A were administered in a different rat (362 g); receptor locations: both in the right lower lobe. *Panel C*, group data of 12 pulmonary C-fibers recorded from 8 rats. FA, fiber activity; B, baseline FA averaged over 10-s interval; LA, peak response (1-s average) after the injection of LA. $^{*}P < 0.05$, significantly different from the baseline. †$P < 0.05$, significantly different from corresponding response obtained during control. (adapted from Ref. [33]).

both jugular C-fiber and nodose Aδ afferents could be activated by acidification (Fig. 3B), dependent upon the magnitude, duration and rate of the pH change [38]. A rapid and transient application of acid stimulated both C and Aδ afferent fibers, whereas a slow and sustained reduction in pH stimulated only the C fibers. Furthermore, the afferent nerve discharge recorded from jugular C-fibers was greater than those from nodose Aδ fibers in response to a given concentration of citric acid (therefore the same pH) (Fig. 3B). Whether these Aδ afferents belonged to a sub-group of the RARs were not known.

In search for "cough receptors", Canning and coworkers identified a specific type of Aδ vagal afferents innervating the extrapulmonary airways (trachea and larynx) with cell bodies residing in the nodose ganglia [39]. Their sensory terminals exhibited exquisite sensitivity to acid challenge and punctuate mechanical stimulation, but was insensitive to capsaicin. These receptors are believed to be primarily responsible for eliciting the cough reflex triggered by acid challenge in the upper airways in anesthetized guinea pigs. Their transduction properties appeared to be different from that of the traditionally defined RARs; for example, they were not activated by lung inflation or bronchoconstriction [39].

4. Ion channels involved in acid-evoked airway irritation

Long before the transient receptor potential vanilloid type 1 (TRPV1) channel was cloned in 1997 [40], a number of studies have shown that low pH solutions evoked a sustained increase of the cation conductance in a sub-population of isolated rat dorsal root ganglion neurons that exhibited capsaicin sensitivity, suggesting a direct effect of hydrogen ion on "capsaicin receptors" on the soma membranes of these neurons [26,27]. This hypothesis was supported by the observations that the stimulatory effect of acid on the vagal and trigeminal C-fiber endings can be abrogated by capsazepine [32,41]. The definitive evidence was established later when Caterina et al. demonstrated that proton can activate and modulate the cloned TRPV1, the "capsaicin receptor", expressed in oocytes [40].

To avoid the possible indirect effects generated by endogenous chemical mediators released from the surrounding tissue or circulating blood, the direct stimulatory effect of hydrogen ion on the airway sensory nerves was studied in isolated rat vagal pulmonary sensory neurons identified by retrograde labeling [42]. When the pH of the perfusing buffer was reduced by steps of 0.5 in the physiological-relevant range (7.0—5.5), different phenotypes of inward current were recorded in these neurons using the whole-cell patch-clamp recording technique (Fig. 4). The percentages of pulmonary sensory neurons responded to step reductions of pH to 7.0, 6.5, 6.0 and 5.5 were 45.2%, 83.1%, 91.5% and 92.5%, respectively. A mild drop of pH to 7.0 evoked only a transient (rapidly activating and inactivating) current with small amplitude in <50% of these neurons. Both the amplitude of this transient current and the percentage of responding neurons began to increase when pH was further lowered (Fig. 4). In addition, a slowly activating and sustained inward current began to emerge when pH was reduced to below 6.5. The current—voltage curve indicated that the transient component of acid-evoked current was carried predominantly by Na^{+}, which was dose-dependently inhibited by amiloride, a known blocker of the acid-sensing ion channels (ASICs) (Fig. 5). The amplitude of the slow, sustained current also increased progressively with lowering pH, and was significantly attenuated by capsazepine, indicating that it was mediated primarily through the activation of TRPV1 (Fig. 5). These two components of acid-evoked current also displayed distinct recovery kinetics from desensitization [42]. A similar observation was reported earlier in isolated rat dorsal root ganglion neurons that acid solution (pH < 6.0) evoked two different types of inward currents [27].

Because there was a clear difference in the pH activation threshold between these two current species (with a higher pH threshold for activating the ASIC-mediated transient current than that for TRPV1), their relative roles in response to a given acid

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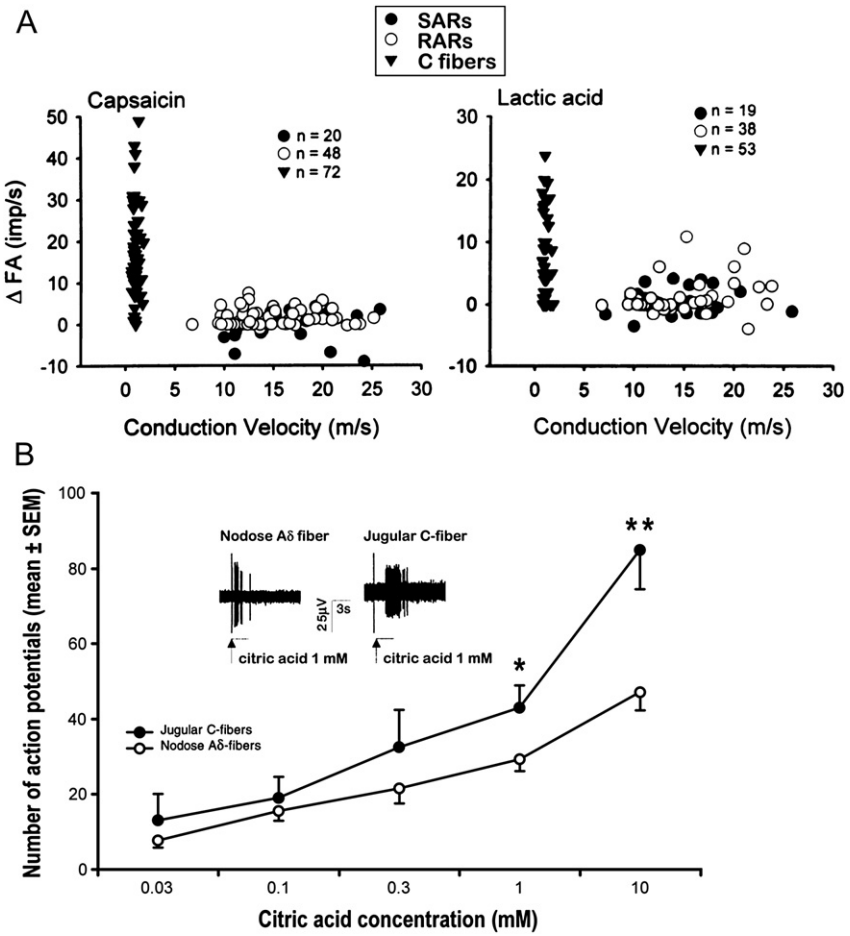

Fig. 3. Stimulatory effects of acid on both C and Aδ afferent fibers innervating the lung and airways in anesthetized rats. *Panel A*, relationship between conduction velocities and fiber activity (FA) evoked by right-atrial bolus injection of capsaicin (1 μg/kg; left) and lactic acid (0.2 mmol/kg; right). ΔFA represents the difference between the peak FA (1-s average) and the baseline FA (averaged over 10-s interval) in each fiber. *Panel B*, concentration–response curve of nodose Aδ-fibers and jugular C-fibers to citric acid. Citric acid was administered as 500 μl volume in 3 s into superfusion over the mechanically sensitive receptive field at 5 min intervals. Each point represents mean ± SE of at least 5 experiments. $^{*}P < 0.05$, $^{**}P < 0.001$. Inset, representative traces of a nodose Aδ fiber (left) and a jugular C-fiber (right) response to citric acid (500 μl, 1 mM). (adapted from Ref. [34,38]).

challenge depended on the level of pH (Fig. 4). The amplitudes of these two different currents varied between neurons, but the difference did not seem to be related to the cell size. In current-clamp recordings, lowering the pH in the same manner caused membrane depolarization and evoked action potentials in these isolated neurons in a proton-concentration dependent manner [42]. Recent studies have shown that the sensitivity of ASIC channels to acid was attenuated as the temperature was raised to body temperature, whereas that of TRPV1 was enhanced by an increase in temperature [43,44]. Therefore, the local tissue temperature is an important factor in determining the relative contributions of TRPV1 and ASICs to the irritant effect of acid in the airways [43]. This difference has important clinical relevance and implication because airway temperature is known to be significantly elevated in asthmatic patients during exacerbation [45].

ASICs are proton-gated ion channels that are voltage-insensitive, and belong to the super family of degenerins/epithelial Na^+ channels [46–48]. They are widely expressed in mammalian sensory neurons. Six different proteins arising from four genes have been cloned to date [49]: ASIC1a and ASIC1b (spliced forms of

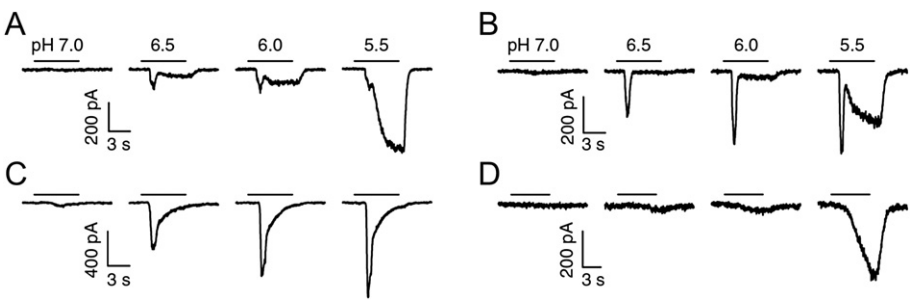

Fig. 4. Representative acid-evoked whole-cell inward currents in isolated rat vagal pulmonary sensory neurons. Acid buffers with increasing proton concentrations were applied for 6 s (horizontal bars) to four different jugular ganglion neurons. The cell capacitances for the neurons in *Panels A*, *B*, *C*, and *D* were 24.2, 23.1, 15.6, and 11.1 pF, respectively. Note distinct sensitivities to low pH and different phenotypes of inward currents in response to acidic challenges. (adapted from Ref. [42]).

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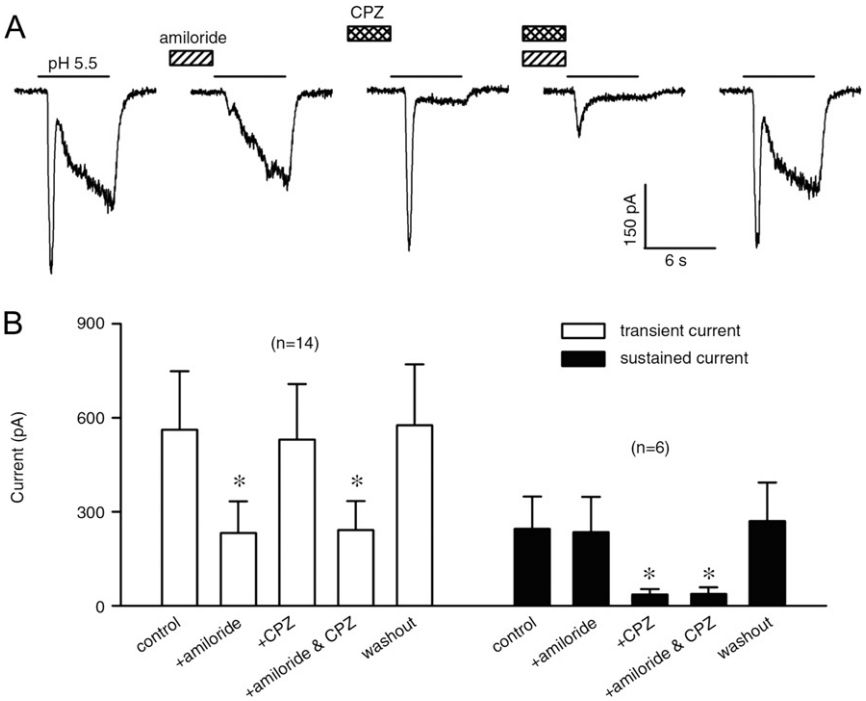

Fig. 5. Effects of amiloride and capsazepine on the acid-evoked currents in rat vagal pulmonary sensory neurons. *Panel A*: representative records illustrating the effects of amiloride (100 μM; 2 min) and capsazepine (CPZ; 10 μM, 2 min) pretreatments on pH 5.5 (6 s)-evoked inward currents in a jugular neuron (23.1 pF). *Panel B*: group data showing the effects of 2-min pretreatment with amiloride (100 μM) and CPZ (10 μM) on both transient and sustained components evoked by low pH (6.5–5.5; 6 s) in the same neurons. $^{*}P < 0.05$ as compared with the corresponding control. Data represent means ± SE. (adapted from Ref. [42]).

the ASIC1 gene), ASIC2a and ASIC2b (sliced forms of ASIC2 gene), ASIC3 and ASIC4. Among them, ASIC2b and ASIC4 do not form functional homomeric channels. However, these subunits can be assembled to form functional heteromeric channel complexes or homomeric channels [49,50], and it is believed that the biphasic current responses (a rapid desensitizing component and a late sustained component) to proton shown by the native nociceptive sensory neurons involve heteromeric ASICs channels (e.g., ASIC2a and ASIC3) [50,51].

TRPV1 is a polymodal transducer, and a tetrameric membrane protein with four identical subunits. Each subunit contains six transmembrane-spanning domains, which form a non-selective cation channel with a high permeability to Ca^{2+} [52]. In the respiratory tract, TRPV1 is expressed predominantly in non-myelinated (C-fiber) afferents [34,53], which represent >75% of the afferent fibers in the pulmonary branch of the vagus nerve [54]. These TRPV1-expressing nerve endings are located near the luminal surface of airway mucosa, either between epithelial cells or forming network-like plexus immediately beneath the basement membrane of epithelium [55], suggesting an involvement of these afferents in regulating the airway responses to inhaled irritants. Activation of these TRPV1-expressing C-fiber sensory terminals triggered centrally-mediated reflex responses that include reflex bronchoconstriction and mucus hypersecretion via the cholinergic pathway, accompanied by the sensation of airway irritation and urge to cough [53]. In addition, activation of TRPV1 also triggers Ca^{2+} influx and a subsequent release of tachykinins and CGRP from the nerve endings, eliciting the local "axon reflex" responses including bronchoconstriction, protein extravasation and inflammatory cell chemotaxis [9].

In a recent study, the expression of messenger RNAs (mRNAs) encoding for TRPV1 and four functional ASIC subunits ASIC1a, ASIC1b, ASIC2a and ASIC3 was detected in rat pulmonary sensory neurons using RT-PCR (Fig. 6). This finding has lent additional support to the electrophysiological evidence that has characterized these two current species involved in the neuronal response to acid challenge [56].

In addition to the vagal sensory neurons described above, expressions of ASIC3 and/or TRPV1 have also been identified in the cell bodies of the dorsal root ganglion neurons (between spinal segments C6-T6) innervating the lung and pleura; either one or both of these two proton-sensitive channels are expressed in these sympathetic afferents arising from the pleura (97%) and the lung (74%) [57]. The more abundant expression of these proton sensors

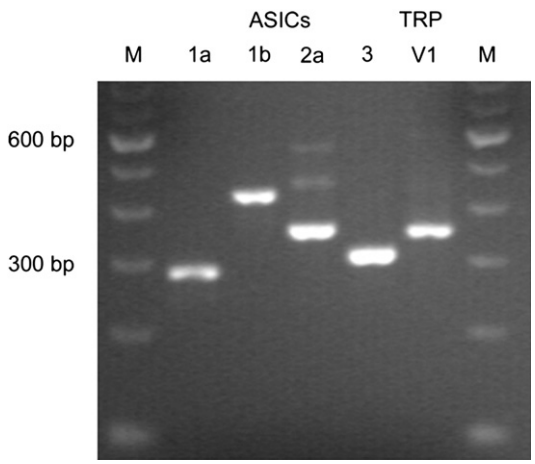

Fig. 6. Detection of the presence of acid-sensing ion channels (ASICs) and transient receptor potential vanilloid receptor 1 (TRPV1) mRNAs in rat pulmonary sensory neurons using RT-PCR. Cytoplasm of 20 individual pulmonary nodose neurons was collected into single PCR tubes. One-step RT-PCR and the nested PCR were carried out to detect the presence of the transcripts for TRPV1 and ASIC subunits 1a, 1b, 2a, and 3. M, DNA molecular weight marker. (adapted from Ref. [56]).

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in the pleural afferents [57,58] may contribute to the high nociceptive sensitivity of the parietal pleura to infection/inflammation during pleurisy [59].

5. Conclusion

When tissue acidification occurs in the respiratory tract under pathophysiological conditions such as inflammation, ischemia and carcinogenesis, it evokes airway irritation, cough and bronchoconstriction. These responses are elicited by the stimulatory effect of hydrogen ion on vagal bronchopulmonary C-fiber and Aδ afferents, which is mediated through activation of two major channel species: ASICs and TRPV1. The distinct difference in the activation and inactivation pattern between these two current species suggests that the ASIC channels are primarily responsible for triggering the sharp response to acid assault in the airways (e.g., acid aspiration), whereas TRPV1 is more involved in the development of slow lingering airway irritation associated with airway disease conditions (e.g., airway inflammation). Recent studies have shown that TRPV1 expression is up-regulated in the sensory nerves innervating the lung and airways during chronic allergic airway inflammation [60] and the laryngeal mucosa in patients with laryngopharyngeal reflux [61]. Furthermore, the responses of isolated pulmonary sensory neurons to acid are enhanced by certain inflammatory mediators [62], such as tryptase [56,63], human eosinophil-derived cationic proteins [64], tumor nacrosis factor α [65], and nerve growth factor [66]. These positive interactions may have important clinical implications because tissue acidification and endogenous release of these chemical mediators often occur concurrently during airway inflammatory reaction [62]. In view of the important role of the TRPV1 expressed on airway sensory nerve terminals in the regulation of the cardiopulmonary function, the increased TRPV1 expression and/or sensitivity to proton may play a part in the manifestation of various symptoms associated with airway inflammatory reaction [53, 67–69].

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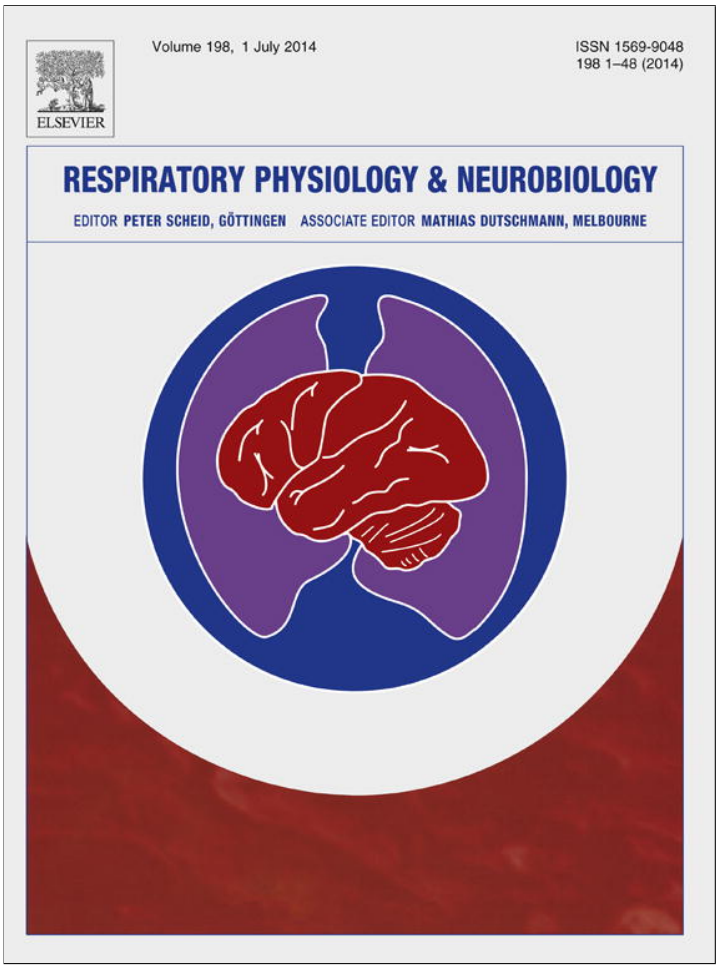

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Breathing hot humid air induces airway irritation and cough in patients with allergic rhinitis

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ABSTRACT

We studied the respiratory responses to an increase in airway temperature in patients with allergic rhinitis (AR). Responses to isocapnic hyperventilation (40% of maximal voluntary ventilation) for 4 min of humidified hot air (HA; 49 °C) and room air (RA; 21 °C) were compared between AR patients ($n = 7$) and healthy subjects ($n = 6$). In AR patients, cough frequency increased pronouncedly from 0.10 ± 0.07 before to 2.37 ± 0.73 during, and 1.80 ± 0.79 coughs/min for the first 8 min after the HA challenge, but not during the RA challenge. In contrast, neither HA nor RA had any significant tussive effect in healthy subjects. The HA challenge also caused respiratory discomfort (mainly throat irritation) measured by the handgrip dynamometry in AR patients, but not in healthy subjects. Bronchoconstriction was not detected after the HA challenge in either group of subjects. In conclusion, hyperventilation of HA triggered vigorous cough response and throat irritation in AR patients, indicating the involvement of sensory nerves innervating upper airways.

1. Introduction

Allergic rhinitis (AR) is an inflammatory disease of upper airways characterized by nasal congestion and rhinorrhea, intermittent or persistent sneezing, pruritus in nose, eyes and throat, and coughing. The inflammatory reaction is characterized by early-phase and late-phase allergic responses similar to that in allergic asthma (Bousquet et al., 2012; Wallace et al., 2008). Repeated exposures to environmental allergens result in an IgE mediated type I allergic response that induces a type-2 helper T cell (TH2) inflammation. Cross-linking of IgE antibodies present on the surface of primed mast cells by an antigen activates them and results in degranulation and release of inflammatory mediators such as histamines, tryptase, and leukotrienes, which in turn leads to vasodilatation and increased vascular permeability. The recruitment of TH2 cells and secretion of IL-5 give rise to tissue eosinophilia that characterizes the late phase response (Middleton et al., 2009). Eosinophilic inflammation in turns can result in further tissue damage and sensitization of the afferent nerves innervating the nose, throat and upper airways due to release of additional inflammatory mediators.

Our laboratory has recently reported that an increase in airway temperature by hyperventilation of hot humid air for 4 min triggered an immediate and transient bronchoconstriction in patients with mild asthma, but not in healthy individuals (Hayes et al., 2012). The bronchoconstriction was accompanied by cough and prevented by pretreatment with ipratropium, a muscarinic receptor antagonist, suggesting an involvement of activation of airway sensory nerves and the cholinergic reflex pathway. Although direct evidence could not be established in that study, our results suggested activation of a temperature sensors expressed in the vagal bronchopulmonary sensory nerves is probably involved in eliciting these reflex responses. One possible candidate is the transient receptor potential vanilloid type 1 receptor (TRPV1). Indeed, chronic allergic inflammation is known to enhance both the sensitivity and the expression of TRPV1 in airway sensory nerves (Lee and Gu, 2009; Zhang et al., 2008).

TRPV1 is also abundantly expressed in the sensory nerve fibers innervating the pharynx, larynx and upper airways (Hamamoto et al., 2008, 2009; Sasaki et al., 2013; Yamamoto and Taniguchi, 2005). However, whether the sensitivity of these TRPV1-expressing sensory nerves is elevated resulting from the chronic inflammation

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of upper airways in AR patients is not yet known, and the reflex responses elicited by an increase in airway temperature in these patients have not been previously studied. This study was therefore carried out to answer these questions.

2. Methods

2.1. Subjects

Adult AR patients and healthy subjects were recruited by public advertisement. A screening interview and a spirometry test were performed in each subject after informed consent was obtained. The diagnosis of AR was confirmed according to the standard clinical guidelines in each patient and a documented positive allergy skin test (Wallace et al., 2008). The American Academy of Allergy, Asthma, and Immunology Joint Task Force on Practice Parameters questionnaire was used to assess and compare symptom severity and global impact of AR in all subjects (Spector et al., 2003). Due to the need to stop therapeutic medications for 2 weeks prior to beginning of the study, patients on steroids and/or have poor AR control were excluded. The study protocol was approved by the Institutional Review Board at the University of Kentucky.

2.2. Isocapnic hyperventilation challenge

A device designed to deliver air of desired temperature and humidity constructed by the University of Kentucky Center for Manufacturing was used as previously described (Hayes et al., 2012). Briefly, a humidified gas mixture of 4.5% CO_2 balanced with air at either hot (HA; 49 °C and 75–80% relative humidity measured by an Extech Hygro-Thermometer, model RH101; Nashua, NH) or room temperature (RA; 20–22 °C and 65–75% relative humidity) was delivered at 300 l/min through a large-bore (7.62 cm) stainless-steel conduit. During the hyperventilation challenge, the subject, while wearing a nose clip, breathed via a mouthpiece into this free stream of humidified gas mixture at ~40% of maximal voluntary ventilation (MVV), determined in each subject in a pre-test, for 4 min; CO_2 was added to maintain an isocapnic condition during hyperventilation. Humidity was generated from sterile isotonic saline by an ultrasonic atomizer (Sonaer Ultrasonics; Farmingdale, NY). The amounts of isotonic saline delivered in RA and HA were 12–14 and 56–60 μl/liter of air, respectively. Humidity and hyperventilation at 40% of MVV were used to facilitate the heat transfer from air to the airway tissue. Levels of end-tidal temperature (model IT-18, Physitemp, Clifton, NJ; time constant: 0.1 s) and CO_2 concentration (Novametrix 1260; Murrysville, PA) were measured before and after 2 min of hyperventilation when these changes reached steady state; and they were measured again at 8 and 16 min after the hyperventilation challenges.

2.3. Pulmonary function measurements

Airway resistance (R_{aw}) was measured continuously by a whole-body constant-volume plethysmography (SensorMedics, Yorba Linda, CA) for 6 min before and 16 min immediately after the hyperventilation challenge. During each measurement, the subject was asked to pant at a frequency of ~2 Hz for ~8 s; R_{aw} was determined by computer, using the center-fit method for the slope measurement within the flow range of ±0.5 l/s. Spirometry test was also performed along with the measurements of other physiological variables (body temperature, heart rate, arterial blood pressure, and oxygen saturation) before and after the challenge.

2.4. Measurement of cough frequency

The number of coughs was recorded manually by listening to and counting the number of explosive cough sounds before, during and after each hyperventilation challenge. A VitaloJAK cough monitor [developed by Vitalograph Ltd. (Lenexa, KS) and the Respiratory Research Group, University of Manchester, UK] was also used in the second half (61%) of the study for a more objective and quantitative measurement of the cough frequency (Smith et al., 2006). The device used a contact microphone placed on the chest wall, a second free field microphone and a custom-made digital recording device to record cough sounds. Cough signals recorded by the cough monitor were played back, and the cough numbers were counted by an individual not familiar with the protocol. Cough frequency measured as number of coughs per minute was then compared with those obtained from manual counting during the experiment; the difference between the data obtained from these two methods was generally less than 10%.

2.5. Measurement of respiratory sensation

Subjects were instructed to indicate the presence and express the degree of respiratory discomfort by squeezing an isometric handgrip dynamometer (model MLT003, ADInstruments; Colorado Springs, CO) with a magnitude of force proportional to the intensity of the sensation felt (Burki et al., 2005; Muza and Zechman, 1984) at intervals of ~2 min following both HA and RA hyperventilation challenges. The resultant voltage generated from the dynamometer transducer was recorded continuously in conjunction with the measurements of R_{aw} and cough responses. To compare the response between subjects, the level of discomfort in each subject was quantified by calculating each response signal as a percentage of the maximum handgrip signal (as 100%) that was determined in each subject before each experiment. After the experiment, the subject was asked to describe verbally if there was any type of respiratory discomfort, and if so, the location of the evoked sensation.

2.6. Study design

HA and RA hyperventilation challenges were given at a random sequence in each subject, usually on two different days. When both challenges were given in the same day, at least 2 h elapsed for recovery. The responses to HA and RA hyperventilation challenges were compared in both AR patients and healthy subjects.

2.7. Statistical analysis

A two-way analysis of variance (ANOVA) was used for the statistical evaluation of the results. When the ANOVA showed a significant interaction, pair-wise comparisons were made with a post hoc analysis (Fisher's least significant difference). Comparisons between the two groups (AR patients vs. healthy subjects) were made using the one-way ANOVA. Data are reported as means ± SEM. P values of <0.05 were considered significant.

3. Results

Seven AR patients between 21 and 43 (35 ± 3) year of age and six healthy subjects between 25 and 48 (32 ± 3) year of age were enrolled in the study; the subject characteristics are shown in Table 1. The AR symptom severity assessment data (Table 1) show that several symptoms with mean scores exceeding 3.5 (out of a total score of 7.0), including sneezing (3.57 ± 0.53; $n = 7$), nasal congestion (5.0 ± 0.58), itchy nose (3.93 ± 0.74), postnasal drip (4.0 ± 0.68), chronic cough (3.57 ± 0.65), eye (3.57 ± 0.53) and ear symptoms (3.57 ± 0.43), were found in AR patients, but none in

Table 1
Subject characteristics and AR symptom severity assessments.

		AR patients							Healthy subjects					
		#1	#2	#3	#4	#5	#6	#7	#1	#2	#3	#4	#5	#6
Physical data	Age (year)	37	40	21	43	41	30	35	25	28	29	32	29	48
	Sex	M	M	F	F	F	F	F	F	M	F	M	M	F
	Height (cm)	180	175	155	150	160	160	165	170	185	157	173	170	160
	Weight (kg)	122	104	59	73	73	53	61	58	86	54	61	84	60
A: Nasal	Sneezing	3	2	5	5	2	5	3	1	1	1	1	1	1
	Runny nose	3	3	4	4	2	5	2	1	2	1	1	2	1
	Congestion (stuffiness)	6	3	6	7	3	5	5	1	1	1	1	2	1
	Itchy nose	6	3	6	3	2	6	2	1	1	1	1	1	1
	Postnasal drip	2	4	6	3	NA	6	3	1	2	1	1	1	1
	Total nasal symptoms	5	5	6	NA	2	6	3	1	2	1	1	1	1
B: Non-nasal	Eye symptoms	6	3	4	3	3	3	3	1	2	1	1	1	1
	Throat symptoms	2	3	6	5	1	NA	3	1	1	1	1	1	1
	Chronic cough	4	1	5	4	2	6	3	1	1	1	1	1	1
	Ear symptoms	3	2	6	3	3	3	5	1	1	1	1	1	1
	Headache	5	1	2	5	1	4	4	1	2	1	1	1	1
	Mental function	1	1	1	5	1	2	1	1	1	1	1	1	1
C:	Quality of life	2	5	3	2	5	2	4	7	7	7	7	7	7

AR: allergic rhinitis; Categories A and B: 1 – none; 7 – unbearably severe; Category C: 1 – none; 7 – excellent; NA: not assessed.

Table 2
Changes in end-tidal (E.T.) temperature and CO_2 concentration caused by hyperventilation of humidified air at room (RA) and high temperature (HA).

	E.T. temperature (°C)		E.T. CO_2 (%)	
	Before	During	Before	During
AR patients ($n=7$)				
RA	33.59 ± 0.29	32.79 ± 0.19*	4.96 ± 0.26	4.94 ± 0.17
HA	33.50 ± 0.31	35.00 ± 0.09*,†	5.14 ± 0.43	5.18 ± 0.47
Healthy subjects ($n=6$)				
RA	33.63 ± 0.35	32.56 ± 0.35*	4.88 ± 0.20	5.15 ± 0.33
HA	33.13 ± 0.27	34.49 ± 0.30*,†	4.93 ± 0.20	5.11 ± 0.24

Measurements were made before and at 2 min after the beginning of the 4-min hyperventilation; the latter was measured immediately after the hyperventilation was interrupted for 3–6 breaths while the subject breathed room air during these measurements.

* Significant difference between before and during the hyperventilation challenge.

† Significant different from the corresponding RA data.

healthy subjects. A comparison between the two groups of subjects showed that there was no significant difference between AR patients and healthy subjects in any of the base-line measurements of physiological variables (Tables 2–4).

In AR patients, hyperventilation of humidified HA generated a significant increase in end-tidal air temperature ($\Delta = 1.5 \pm 0.2$ °C; $P < 0.05$, Table 2). In contrast, hyperventilation of RA decreased the end-tidal temperature significantly ($\Delta = 0.8 \pm 0.24$ °C; $P < 0.05$, Table 2) in the same patients. Hyperventilation of either HA or RA did not change the end-tidal CO_2 concentration in these patients (Table 2). Similar changes were also recorded in healthy subjects (Table 2).

The HA challenge consistently triggered cough in AR patients (e.g., Fig. 1). Number of coughs was 0.10 ± 0.07 coughs/min at baseline, increased to 2.37 ± 0.73 coughs/min ($P < 0.01$, $n = 7$) during the isocapnic hyperventilation of HA, and subsequently to 1.80 ± 0.79 coughs/min ($P < 0.01$, $n = 7$) in the first 8 min following the HA challenge (Fig. 2). In contrast, hyperventilation of RA did not cause any significant tussive effect in the same patients (Fig. 2). In two of the AR patients, the cough responses to the HA challenge were compared in separate experiments when the humidity of HA were generated from isotonic saline and distilled water, and similar responses were found; cough frequencies were 4.7 ± 0.5 and 4.1 ± 0.4 coughs/min ($n = 2$) during the HA challenges with the humidity generated from saline and distilled water, respectively.

AR patients expressed a significantly higher degree of respiratory discomfort via handgrip dynamometry after the hyperventilation of humid HA (Fig. 3). Their hand grip signal increased to 18.8 ± 6.9% of the maximum hand grip signal after humid HA challenge compared to 8.9 ± 3.7% after RA hyperventilation ($P < 0.05$, $n = 7$). In AR patients, verbal description of respiratory discomfort after the experiment included “throat irritation and tickling”, and “dry and sore throat.” The location of irritation was described by these patients as mainly in or below the larynx area.

In AR patients, the FEV_1/FVC ratio decreased significantly from 82.1 ± 2.1% to 78.3 ± 1.4% at ~8 min after the HA challenge ($P < 0.05$, $n = 7$); FEV_1 and FVC, however, did not change significantly and remained within normal range (Table 3). The small

Table 3
Changes in forced expiratory volumes caused by hyperventilation of humidified air at hot (HA) and room temperature (RA).

	FEV_1 (l)		FVC (l)		FEV_1/FVC	
	Before	After	Before	After	Before	After
AR patients ($n=7$)						
RA	3.12 ± 0.28	2.99 ± 0.25	3.84 ± 0.35	3.78 ± 0.32	81.26 ± 2.01	79.44 ± 1.37
HA	3.12 ± 0.30	3.08 ± 0.30	3.82 ± 0.37	3.92 ± 0.36†	82.09 ± 2.12	78.31 ± 1.44*
Healthy subjects ($n=6$)						
RA	3.67 ± 0.32	3.63 ± 0.32	4.50 ± 0.44	4.54 ± 0.44	82.07 ± 2.26	80.43 ± 2.56
HA	3.66 ± 0.33	3.63 ± 0.33	4.46 ± 0.46	4.53 ± 0.49	82.52 ± 2.39	80.96 ± 2.47

Forced expiratory tests were performed before and at ~8 min after the hyperventilation challenge in allergic rhinitis (AR) patients and healthy subjects.

* Significant difference between before and during the hyperventilation challenge.

† Significant different from the corresponding RA data.

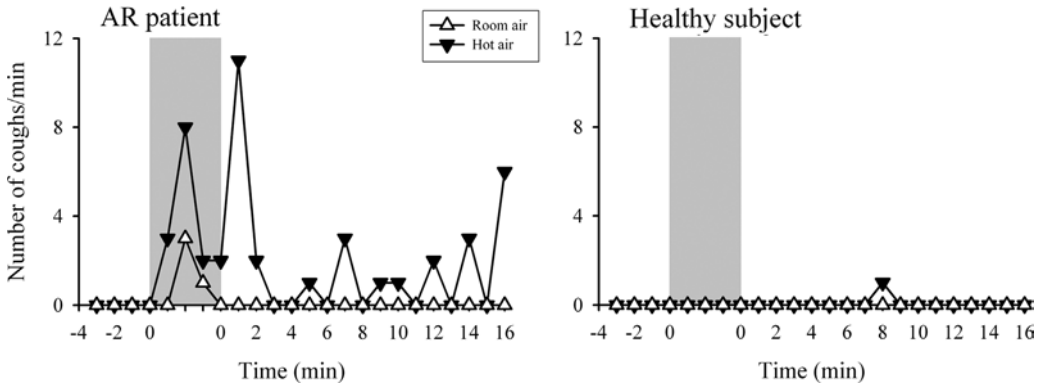

Fig. 1. Representative responses of cough frequency (number of coughs per minute) to hyperventilation of humidified room air (open triangles) and hot air (closed triangles) in an AR patient (*left panel*) and a healthy subject (*right panel*). During hyperventilation (shaded bars), the subjects breathed at 40% of maximal voluntary ventilation for 4 min of a gas mixture of 4.5% CO_2 balance air.

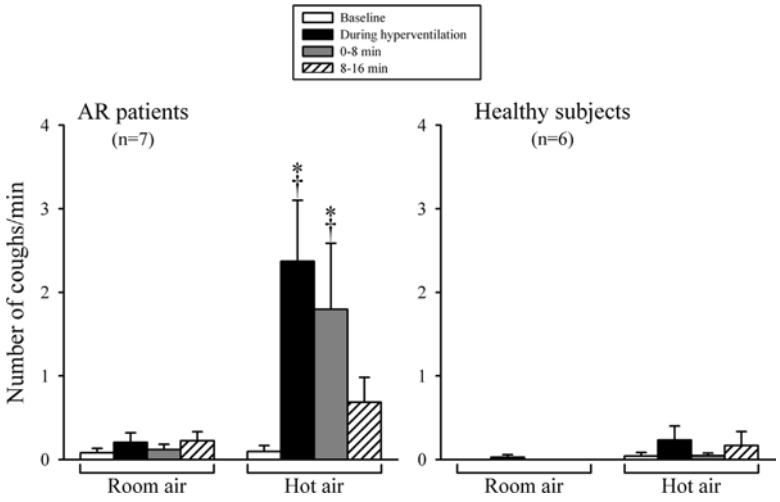

Fig. 2. Comparison of cough responses to hyperventilation of humidified room air and hot air in AR patients (*left panel*) and healthy subjects (*right panel*). Cough frequencies were averaged in 8-min durations before and after hyperventilation challenge in each subject. Data are means ± SEM. *Significantly different from the baseline. †Significant difference between room air and hot air.

but significant decrease in the FEV_1/FVC ratio seemed to indicate a mild bronchoconstriction, which however was not detected by the measurement of R_{aw} in these patients (Fig. 4). On average, R_{aw} did not significantly change after the HA challenge in AR patients: $R_{aw} = 2.08 \pm 0.18\ cmH_2O/L/s$ at baseline; the peak $R_{aw} = 2.76 \pm 0.28\ cmH_2O/L/s$ after the HA challenge ($P > 0.05$, $n = 7$). Heart rate increased slightly but significantly during the HA challenge from 74.6 ± 3.9 to 82.6 ± 4.2 beats/min ($P < 0.05$, $n = 7$) (Table 4). AR patients did not develop any wheezing during and after either HA or RA challenge.

Healthy subjects, in a sharp contrast to that in AR patients, did not exhibit significant tussive response during isocapnic HA hyperventilation (Figs. 1 and 2). Healthy individuals also described a subtle feeling of dry throat during the HA hyperventilation challenge (despite the 75–80% relative humidity in the HA). However, none of the healthy subjects expressed throat irritation or respiratory discomfort (Fig. 3). R_{aw} did not change significantly after the HA challenge in healthy subjects, similar to that in AR patients (Fig. 4); FEV_1, FVC and FEV_1/FVC ratio did not change significantly, either ($P > 0.05$, $n = 6$) (Table 3). Heart rate increased significantly during

Table 4
Effects of hyperventilation of humidified air at hot (HA) and room temperature (RA) on other physiological variables.

	Body temperature (°C)		Sys. B.P. (mmHg)		Dia. B.P. (mmHg)		Heart rate (bpm)		O_2 saturation (%)	
	Before	During	Before	During	Before	During	Before	During	Before	During
AR patients ($n = 7$)										
RA	36.5 ± 0.2	35.8 ± 0.2*	124.7 ± 5.9	126.4 ± 7.6	77.3 ± 5.6	78.0 ± 5.6	76.3 ± 5.2	79.7 ± 5.8	97.6 ± 0.5	97.7 ± 0.6
HA	36.3 ± 0.2	36.4 ± 0.2†	124.6 ± 6.9	122.1 ± 5.9	74.6 ± 4.0	80.1 ± 4.6*	74.6 ± 3.9	82.6 ± 4.2*	97.9 ± 0.6	98.0 ± 0.5
Healthy subjects ($n = 6$)										
RA	36.5 ± 0.2	35.8 ± 0.2*	129.3 ± 7.2	130.8 ± 7.1	76.2 ± 3.9	74.8 ± 4.2	67.5 ± 5.6	69.5 ± 5.2	97.8 ± 0.7	98.5 ± 0.7*
HA	36.2 ± 0.2†	36.4 ± 0.1*†	131.3 ± 6.4	129.2 ± 6.0	76.2 ± 3.5	79.0 ± 3.3†	66.0 ± 5.3	70.3 ± 5.6*	98.7 ± 0.6†	98.8 ± 0.5

Measurements were made before and at 2 min after the beginning of the 4-min hyperventilation in AR patients and healthy subjects.
* Significant difference between before and during the hyperventilation challenge.
† Significant different from the corresponding RA data.

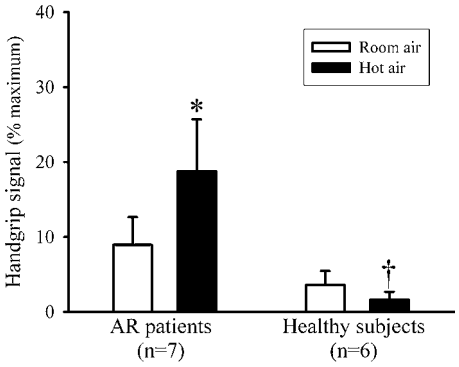

Fig. 3. Airway irritation evoked by hyperventilation of humidified room air (open bars) and hot air (closed bars) in AR patients and healthy subjects. The level of discomfort sensation was expressed by the handgrip dynamometer signal in each individual. *Significant difference between room air and hot air. †Significant difference between AR patients and healthy subjects.

the HA challenge from 66.0 ± 5.3 to 70.3 ± 5.6 beats/min ($P < 0.05$, $n = 6$), similar to that in AR patients (Table 4). Wheezing was not detected in any of the healthy subjects during and after either HA or RA hyperventilation.

4. Discussion

The results of this study showed that an increase in airway temperature triggered vigorous cough responses and evoked throat irritation in AR patients, but not in healthy individuals. We chose the protocol of hyperventilation of humidified HA to increase the airway temperature because it has been well illustrated that both hyperventilation and the humidity in the HA can facilitate the delivery of "heat load" from the inhaled HA to the airway tissue (Aitken and Marini, 1985). However, we can dismiss hyperventilation as a contributing factor to the tussive effect because hyperventilation of humid RA did not generate cough or throat irritation in the same AR patients. We used isotonic saline to humidify the inspired gas mixture in this study because inhalation of distilled water aerosol is known to trigger bronchoconstriction and cough in patients with asthma, whereas isotonic saline aerosol did not in the same patients (Sheppard et al., 1983). This excitatory effect of distilled water is believed to result from a stimulation of laryngeal sensory nerves and/or rapidly adapting airway receptors due to the low concentration of chloride ion and/or the low osmolarity in distilled water (Anderson et al., 1990; Pisarri et al., 1992). However, when the HA challenges generated from isotonic saline and distilled water were tested separately in two AR patients in this study, their cough responses to saline and distilled water were not different, which may be related to the relatively low water content delivered in the humid HA in our study compared with that delivered in aerosol in the study by Sheppard and coworkers (1983). Furthermore, in a recent study in allergen-sensitized rats, when the same water content as that contained in the humidified HA was delivered by aerosolized saline at room temperature, it failed to generate any significant airway responses (Hsu et al., 2013). In addition, in the present study hyperventilation of humidified RA, despite presence of atomized saline, did not induce any significant cough response in the same AR patients (Fig. 2). Thus, although we cannot dismiss the role of humidity in the delivery of "heat load" (Aitken and Marini, 1985) in this study, the collective evidence suggests that the tussive response and throat irritation were caused primarily by the increase in airway tissue temperature generated by the HA hyperventilation.

In addition to the tussive effect, hyperventilation of HA also evoked a significantly higher degree of respiratory discomfort, compared to the RA hyperventilation challenge; in this study we used a hand-grip dynamometer for subjects to express the respiratory discomfort (Burki et al., 2005). The device functions on the basis of Stevens' psychophysical power law that states the perceived magnitude of sensation relates exponentially to the level of the stimulus (Stevens, 1957). It is a cross-modality matching instrument that matches subjects muscle force to their perceived level of discomfort, similar to the magnitude estimation by numerical scale. In fact, the two modalities have been shown to be equivalent in scaling respiratory sensation (Muza and Zechman, 1984). Verbal descriptions of the discomforts in AR patients uniformly pointed to throat irritation and tickling, and the sites of irritation were localized in the larynx area. In contrast, throat irritation was not detected or expressed by any of the healthy individuals despite receiving the same hyperventilation of HA challenge. All these information and evidence seem to suggest the possible involvement of stimulation of sensory nerves innervating the larynx, laryngopharynx and/or upper airways in the AR patients.

The specific types of sensory nerves and temperature sensors that are responsible for evoking the sensation of throat irritation and cough during and after the HA hyperventilation challenge in AR patients cannot be identified in this study. However, some of the potential candidates should be considered. The primary sensors

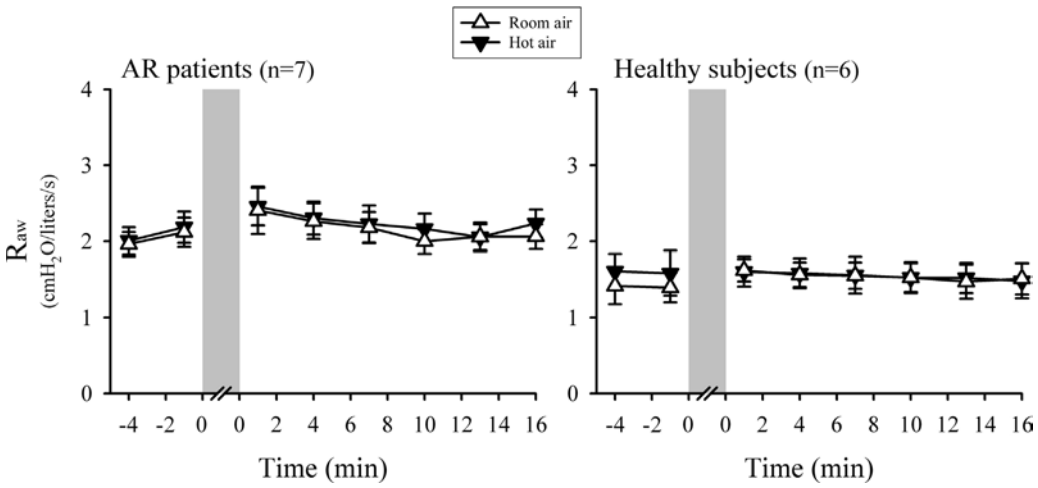

Fig. 4. Comparison of responses of airway resistance (R_{aw}) to hyperventilation (shaded bars) of humidified room air (open triangles) and hot air (closed triangles) in AR patients (*left panel*) and healthy subjects (*right panel*). Each data point represents R_{aw} averaged over 4 consecutive breaths, and data are means ± SEM of all subjects in that group.

for detecting warm and hot temperature in mammalian species are TRPV channels (Nilius et al., 2007). TRPVs are a family of ion channels containing six trans-membrane domains that form non-selective, non-voltage-gated cationic channels (Nilius et al., 2007). Each of the four subtypes of TRPVs (TRPV1–4) is activated in a different temperature range (Benham et al., 2003; Dhaka et al., 2006). The increase in airway temperature generated by HA challenge in the present study likely activated more than one type of these TRPV channels. Among them, the potential involvement of TRPV1 merits further consideration. TRPV1 is abundantly expressed on the nerve terminals of the vagal and non-vagal C-fiber afferents innervating the entire respiratory tract, including the larynx, pharynx and upper airways (Hamamoto et al., 2008, 2009; Lee and Yu, 2014; Sasaki et al., 2013; Yamamoto and Taniguchi, 2005). Using the whole-cell perforated patch clamping technique, our laboratory has recently demonstrated that an increase in temperature within the normal physiological range (35–41 °C) evoked inward currents (in voltage-clamp mode), and membrane depolarization and action potentials (in current-clamp mode) in isolated vagal pulmonary sensory neurons (Ni et al., 2006). These responses were reduced by >50% after a pretreatment with a selective antagonist of the TRPV1 channel, AMG 9810. This observation is of particular importance because it demonstrated that this effect is mediated primarily through activation of the TRPV1 channel (Ni et al., 2006). More importantly, the bronchopulmonary sensory neurons could be activated by increasing temperature to the levels considerably lower than 43 °C, the temperature that was originally reported as the temperature threshold for activating the heterologously expressed TRPV1 receptor (Caterina et al., 1997).

In this study, the same HA challenge did not evoke cough or throat irritation in healthy subjects. The difference in these responses between AR patients and healthy individuals could probably be related to the fact that the TRPV1 sensitivity can be elevated in the presence of tissue inflammation because endogenous inflammatory mediators such as bradykinin, prostaglandins, and nerve growth factor are known to cause post-translational sensitization of TRPV1 receptor (Shin et al., 2002; Sugiura et al., 2002; Zhang et al., 2005). In addition, our laboratory recently reported that chronic allergic inflammation in Brown-Norway rats actively sensitized with ovalbumin induced a significant increase in the expression of TRPV1 in bronchopulmonary neurons in nodose ganglia, mainly in neurofilament-positive (myelinated) neurons (Zhang et al., 2008). Indeed, the sensitivity to capsaicin, a selective activator of TRPV1, was detected in some of the vagal myelinated (A-fiber) afferents that normally do not exhibit capsaicin sensitivity (Zhang et al., 2008). Our hypothesis is supported by the study of Pecova and coworkers who have reported a significantly higher cough sensitivity to capsaicin in patients with seasonal allergic rhinitis compared to healthy individuals (Pecova et al., 2008). Whether an up-regulation of the TRPV1 expression in the afferent fibers innervating larynx, laryngopharynx and upper airways in the AR patients remains to be investigated, nonetheless.

In our current study the pronounced tussive effect generated by the HA challenge was not accompanied by any increase in R_{aw} in AR subjects. These results are different from the significant bronchoconstrictive responses to HA challenge that we recently reported in patients with mild asthma (Hayes et al., 2012). It is known that chronic inflammation in asthma results in hypertrophy and hyperplasia of airway smooth muscles, subepithelial fibrosis, increase in mucus glands and vascularity throughout the entire tracheobronchial tree, a process known as airway remodeling (Fahy et al., 2000). The chronic inflammation known to occur in the lower airways of asthmatics and the resultant release of inflammatory mediators and cytokines can lead to the hypersensitivity of bronchopulmonary C-fiber afferents (Lee and Yu, 2014), which may further enhance the bronchoconstrictive response in asthmatic patients by causing additional reflexive contraction of hypertrophic airway smooth muscles through the cholinergic pathway (Hayes et al., 2012). Thus, we postulate that the discrepancy between these two studies is related to the differences in the site and degree of chronic inflammation, and the subsequent post-inflammatory changes between these two patient groups.

In this study we did not have the data to determine the temperature threshold for triggering the cough responses, which precludes us from any speculation regarding the environmental conditions (temperature and humidity) that can cause worsening of symptoms in AR patients. Although the temperature of 49 °C used in our HA challenge is relatively high compared to the range of environmental temperature, the HA challenge only generated a small increase in the end-tidal temperature plateau in both AR and healthy subjects in this study (Table 2). Furthermore, it is conceivable that the same increase in airway tissue temperature can be generated by breathing hot humid air at a lower temperature for a longer duration (>4 min). More importantly, the hyperventilation at the level of 40% of MVV simulates the breathing during light to moderate levels of exercise when subjects breathe through mouth instead of nose. Hence, based upon our findings in this study, it seems reasonable to postulate that exercise tolerance of AR patients may be adversely affected in hot and humid environments.

In summary, this study clearly demonstrated that hyperventilation of humid HA triggered vigorous cough responses and evoked throat irritation in AR patients, but not in healthy individuals. Furthermore, these tussive and irritating effects were not detected in the same AR patients after hyperventilation of humid RA. Although the mechanisms promoting these responses are not yet fully understood, these findings pointed to a possible involvement of activation of the thermal sensors expressed in the sensory nerves innervating larynx, laryngopharynx and upper airways by an increase in airway temperature.

Funding

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Acknowledgements

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Sensory Nerves in Lung and Airways

Lu-Yuan Lee[*1] *and Jerry Yu*[2,3,4]

ABSTRACT

Sensory nerves innervating the lung and airways play an important role in regulating various cardiopulmonary functions and maintaining homeostasis under both healthy and disease conditions. Their activities conducted by both vagal and sympathetic afferents are also responsible for eliciting important defense reflexes that protect the lung and body from potential health-hazardous effects of airborne particulates and chemical irritants. This article reviews the morphology, transduction properties, reflex functions, and respiratory sensations of these receptors, focusing primarily on recent findings derived from using new technologies such as neural immunochemistry, isolated airway-nerve preparation, cultured airway neurons, patch-clamp electrophysiology, transgenic mice, and other cellular and molecular approaches. Studies of the signal transduction of mechanosensitive afferents have revealed a new concept of sensory unit and cellular mechanism of activation, and identified additional types of sensory receptors in the lung. Chemosensitive properties of these lung afferents are further characterized by the expression of specific ligand-gated ion channels on nerve terminals, ganglion origin, and responses to the action of various inflammatory cells, mediators, and cytokines during acute and chronic airway inflammation and injuries. Increasing interest and extensive investigations have been focused on uncovering the mechanisms underlying hypersensitivity of these airway afferents, and their role in the manifestation of various symptoms under pathophysiological conditions. Several important and challenging questions regarding these sensory nerves are discussed. Searching for these answers will be a critical step in developing the translational research and effective treatments of airway diseases. Published 2014. *Compr Physiol* 4:287-324, 2014.

Introduction

Sensory nerves in the respiratory tract play an essential role in regulating two of the primary functions of the respiratory control system: (i) maintenance of homeostasis by regulation of depth and rate of breathing, bronchomotor tone, airway secretion, and other cardiopulmonary functions under both healthy and disease conditions; (ii) initiation of important defense reflexes that protect the lung and body from potential health-hazardous effects of air-borne particulates and chemical irritants (65, 283, 324, 385). The afferent activities arising from the lung and airways are conducted primarily by branches of vagus nerves that innervate the entire respiratory tract ranging from larynx to lung parenchyma, and project to the nucleus tractus solitarius (NTS) in the medulla. These sensory fibers represent approximately one-fifth of the total number of afferent fibers carried in the vagus nerves (179). Signals arising from the pulmonary structures are also conducted by afferent fibers contained in the sympathetic nerves via the white rami communicants to the spinal cord. The specific role of these "sympathetic afferents" in the regulation of respiratory functions is not as clearly defined.

Several comprehensive and in-depth reviews on the physiological functions and properties of these sensory nerves were published over three decades ago (65, 283, 324, 385). This review focuses primarily on recent findings on receptor structures, transduction properties and reflex functions of these sensory nerves, and the mechanisms involved in the regulation of their sensitivity. Table 1 outlines the major topics covered in this review.

Vagal Afferents Innervating the Respiratory Tract

Receptor subtypes and afferent properties

Sensory receptors can be classified in different ways. According to sensory modalities and anatomic location, sensory receptors can be divided into three groups in mammals: special sense, somatic and visceral. Visceral receptors carry information concerning the body's internal environment—such as pressure, osmolarity, temperature, etc. Visceral sensory fibers, including those innervating the respiratory tract, travel

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Table 1 Major topics presented in this review on properties and functions of sensory nerves in the lung and airways

- Vagal afferents innervating the respiratory tract
  - Receptor subtypes and afferent properties
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- Important unanswered questions

with autonomic nerves such as the sympathetic nerves and the vagus nerves. These sensory receptors can be characterized anatomically by location (e.g., laryngeal vs. bronchiolar), physiologically by sensory modality (e.g., mechanosensitive vs. chemosensitive), morphologically by structures (e.g., plant-like vs. oval shaped), and chemically by molecular or chemical composition (e.g., substance P immune reactive vs. Na^+/K^+-ATPase immune reactive). A good categorization system should identify a sensor comprehensively from all aspects. However, our present knowledge about the sensory receptor morphology and molecular composition is deficient. Most research is carried out through physiological means, and a combination of anatomical and physiological classification is commonly adopted. Assessed by conduction velocity, two types of afferents nerves supplying vagal sensory receptors can be distinguished: nonmyelinated and myelinated.

Laryngeal afferents

In view of the strategic location and critical role of larynx in the protective and regulatory functions of breathing, it should not be surprising that the larynx is supplied by a substantially larger number of sensory nerves per unit of lumenal surface area than anywhere else in the entire respiratory tract (330). The larynx is innervated by the recurrent laryngeal nerves and by the internal and external branches of superior laryngeal nerves. The cell bodies of these laryngeal afferents are located in the two vagal sensory ganglia, with a majority of them in the nodose ganglion (387). Afferent properties and functional characteristics of these receptors have been extensively studied and clearly documented (329). Sant'Ambrogio and co-workers have classified the laryngeal afferents that exhibit respiratory-related activities into three major categories based upon their sensory modality (Fig. 1), and their activities are conducted in myelinated afferents that represent the majority of the laryngeal afferents (327, 329). Cold receptors sense the drop in temperature. Because the drop of laryngeal lumenal temperature is proportional to the magnitude of inhaled airflow, these receptors are sensitive to inspired flow and were, therefore, initially named "flow" receptors (327). Drive receptors are activated by the contraction of laryngeal muscles during respiratory movements. Pressure receptors detect either negative (collapsing) or positive (distending) transmural pressure in the larynx during respiration (Fig. 1), and are the dominant subtype of these laryngeal afferents. Since these pressure receptors are located in the most compliant region of the upper airways, they are in an ideal position to detect the abnormal collapsing pressure that occurs in the situations such as obstructive sleep apnea. Stimulation of these receptors can initiate reflex responses including activation of upper airway dilating (abductor) muscles and inhibition of inspiratory muscle drive; both actions prevent further collapsing and thereby preserve the patency of the upper airway in wakefulness and sleep (93, 152, 250, 329). A recent article has provided a more in-depth review of the important role of these laryngeal afferents in the reflex regulation of laryngeal caliber and central respiratory drive (166).

The other major type of laryngeal afferents exhibits no or irregular spontaneous activity that is unrelated to the respiratory cycles (329). They consist of both myelinated and unmyelinated afferents. These receptors have distinct sensitivity to chemical and mechanical stimulation (30, 216) and are responsible for eliciting the protective reflex responses (e.g., apnea, cough, etc.) against inhaled irritants (329). Some of these afferents can be activated by high concentration (>8%) of CO_2 (30, 216). These receptors are also stimulated when they are exposed to the solution lacking permanent anions (e.g., chloride ion) administered either by topical application or by aerosol (329), which may be partially responsible for the apneic response to inhalation of distilled water aerosol

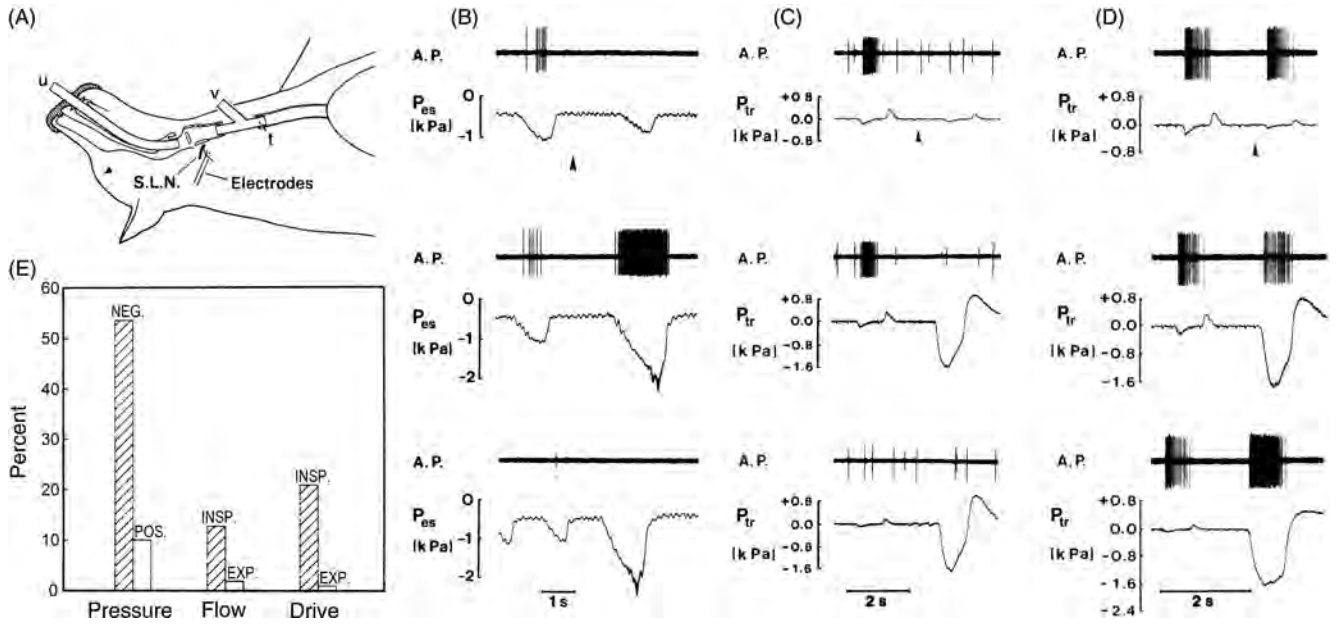

Figure 1 Laryngeal sensory receptors exhibiting respiratory-related activities. (A) A schematic drawing of the experimental setup for studying laryngeal afferents in anesthetized dog. Mouth and nares are sealed around tube (u). When the side arm of the tracheal cannula (v) is occluded, the dog breathes through the upper airway: when v is open and u occluded the dog breathes through the tracheostomy, upper airway occlusion is performed by closing both u and v, and tracheal occlusion by obstructing the tracheal cannula at (t) by inflating the cuff of a Foley catheter. Electrodes record the action potentials from "single" fibers isolated from the peripheral cut end of the internal branch of the superior laryngeal nerve (S.L.N.). In all records (B, C, and D), upper panel: upper airway breathing changed to tracheostomy breathing at the arrow; middle panel: upper airway breathing and upper airway occlusion; lower panels: tracheostomy breathing and tracheal occlusion. (B) A laryngeal "pressure" receptor responding to negative transmural pressure. Note that maximal activity is seen during upper airway occlusion, in which the larynx is subjected to increased negative pressure. (C) A cold ("flow") receptor (see explanations in the text). Note that a phasic inspiratory activity is present only when air flows through the larynx. (D) A "drive" receptor responding to distortion due to the action of upper airway muscles. Note that an inspiratory activity is present and is not influenced by flow. The inspiratory activity and duration increase when the inspiratory "drive" increases in response to airway occlusion. (E) Distribution (percentage) of the three types of laryngeal receptors recorded in this study ($n = 110$). Note that pressure receptors are the most frequently occurring type. A.P., action potentials; Pes, esophageal pressure; Ptr, tracheal pressure; kPa, kilopascal. [Modified, with permission, from reference (327).]

in humans (96, 275). The transduction mechanism(s) is not fully understood, but the responses seem to be evoked by either abnormal ionic compositions or low osmolality of the liquid layer lining the laryngeal mucosal surface (329).

Bronchopulmonary afferents

Bronchopulmonary afferents are connected with two major types of receptors or sensors: mechanosensors and chemosensors. They continuously transmit the information regarding changes in physical condition and chemical environment in the airway and lung tissues to the central nervous system (CNS) (65). Extrapulmonary sensors are mainly perfused by bronchial circulation and intrapulmonary ones largely by pulmonary circulation (229). In addition, large and small airways are subjected to different mechanical forces during ventilatory cycles. Thus, sensors in different regions may serve different functions, and this is an area lacks exploration. Conventionally, sensory receptors are identified by single unit recordings developed by Adrian in 1933 (3). In 1946, Knowlton and Larrabee divided the mechanosensors into rapidly adapting receptors (RARs) and slowly adapting receptors (SARs) based on adaptation rate to constant-volume lung inflation (191). Adaptation rate is often expressed as the difference between the peak discharge frequency of the first s and the average frequency of the second s, which is then divided by the peak frequency of the first s and multiplied by 100. The adaptation rates above 70% and below 50% are considered as rapidly and slowly adapting, respectively (403). Receptors with a rate between 50% and 70% are usually not studied or referred to as intermediate adapting receptors. These mechanosensors were intensively studied by Widdicombe and Sant'Ambrogios in the following decades. Pioneered by Paintal and Coleridges, C-fiber receptors in the airways were identified and characterized by their response to chemical stimulants delivered intravenously or by aerosol. The afferent properties and reflex functions of C-fibers have been extensively studied and reviewed (65, 286). Since the last comprehensive review of this topic in the Hnadbook of Physiology more than 25 years ago (65), new information indicates that the classification needs expansion. In addition to SARs (332), RARs (330) and C-fibers (225), deflation-activated receptors (DARs) and high threshold Aδ receptors (HTARs) have been identified and included in this review. Conventionally, DARs are considered as a sub-type of RARs. Such a view point has been challenged (403). For the reasons discussed in pages 10-12, we will discuss DARs as an individual type of mechanosensors.

Slowly adapting receptors Location and distribution. Reports on whether SARs are mainly distributed in the extrapulmonary or intrapulmonary airways vary (65). However, most physiological and morphological studies indicate that SARs are distributed along the airways with the density being highest in the trachea and gradually decreasing along the airways to the lung periphery (16, 324, 403). In the trachea, they are located in the posterior wall and many are embedded in the smooth muscle layer. In the peripheral airways they are found in the entire airway wall. A single SAR unit may contain several receptive fields (see pages 10-12).

Afferent properties. SARs are mechanosensors and generally insensitive to chemicals. They exist in a variety of species including the human (403) and are found to be active at birth (101). SARs are the best characterized among the airway sensors, because they can be easily identified and recorded due to their regular discharge patterns and large amplitude of action potentials. During eupneic breathing, they discharge regularly, characterized by increases during lung expansion and decreases during lung deflation. Activation of SARs by increased lung volume and inflation pressure is probably due to an increase in the airway wall tension. They sense sustained changes, although may also possess dynamic properties (325). They adapt very slowly to maintained lung inflation, and the activity can sustain for as long as an hour (76). Some SARs have low-threshold and discharge throughout the respiratory cycle (tonic); others have high-threshold and discharge only during lung inflation (phasic) (65). Low-threshold SARs are found more frequently in central airways, whereas high-threshold ones are more in peripheral airways (310). However, some low-threshold SARs can be converted to high-threshold when lung compliance decreases, but the pattern is restored after compliance returns. Based on the pattern of their response to lung inflation, SARs are also described as two types: Type I discharge plateaus at inflation pressures above 10 cmH_2O, whereas Type II activity increases linearly beyond 30 cmH_2O. Type I and type II receptors were suggested to operate in parallel and in series with smooth muscle fibers, respectively (261). This classification and its explanation have been challenged (277). Although some SARs may have a response plateau, it can be any pressure value applied. Now, we know that the sensing range of a sensory unit depends on receptor composition of the unit (see page 11).

SARs are usually not activated directly by chemical agents. Due to the mechanosensitivity of SARs, their activity can be modified by chemical agents that alter the mechanical properties of the airways and lung. For example, SARs are stimulated by airway constriction induced by inhalation of nicotine (356). Instillation of surfactant decreases lung stiffness and also lowers SAR activity (54). It is interesting that allergen-induced airway inflammation causes vagal myelinated afferents (including SARs and other receptors) to respond to capsaicin, which is supported by a pronounced increase of expression of the transient receptor potential vanilloid type 1 (TRPV1) receptor in large-diameter sensory neurons in nodose ganglia. The effects of endogenous chemical mediators including neurotrophic factors are believed to be involved in the process (418).

Rapidly adapting receptors Location and distribution. As SARs, RARs are distributed along the airways with a high density in the large airways. They are believed to be abundant in the carina (65). In the trachea, they are found in the entire circumference of the tracheal wall. Some sensory units identified as RARs can be activated by light touching of respiratory mucosa and by lung inflation. Removing mucosa eliminates the touching response but not the inflation response (328). Thus, RARs are thought to have multiple sensory endings in the different layers of the airway wall.

Afferent properties. RARs, by definition, are mehanosensors. In contrast to SARs, they sense the dynamic changes in lung volume and mechanics. They are stimulated mainly by the rate of change in the amplitude of stimulation. In quiet breathing, many RARs are inactive and others discharge irregularly at a low activity with less than 1 impulse/s (65). However, more activity occurs during the lung inflation phase. RARs respond to changes in lung volume, flow rate, airway pressure, the rate of change of airway pressure (dp/dt), and lung stiffness (65, 330). These stimuli are interrelated and sometimes difficult to differentiate one from the other. In anesthetized open-chest dogs mechanically ventilated at constant rate and tidal volume, RAR activity increased step wisely and discharged vigorously during lung inflation, as lung stiffness step wisely increased by briefly removing PEEP (183). RAR activity also increased when the flow rate, and hence dp/dt, was increased, while keeping tidal volume and ventilatory cycle constant. At the same dp/dt, RARs were stimulated more by increasing stiffness than by increasing inflation rate. The effects of lung stiffness on RARs are also observed in spontaneous breathing dogs (298) and in open-chest cats and rabbits (406). These studies indicate among tidal volume, airway pressure, airflow rate, dp/dt, and lung stiffness, increased lung stiffness is the most effective stimulus.

In addition to a direct effect, chemicals can stimulate RARs through a secondary effect by their effects on lung mechanics. RARs were stimulated by right atrial injections of histamine in monkeys (313), cats (409), and guinea pigs (22). The stimulation was associated with an increase in tracheal pressure. The secondary mechanical effects on RARs are frequently observed in other experiment models. Intravenous administration of NaCN led to increases in lung stiffness and RAR activity in the rabbit (254). Pulmonary air embolism also stimulates RARs by secondary mechanical effects in dogs (53). The secondary effects were also reported with ozone in dogs (66), bradykinin in guinea pigs (22), cigarette smoke in dogs (201), and endotoxin in rats (208).

Historically, RARs have a variety of names, including "deflation receptors," "cough receptors," "pulmonary flow receptors," and "irritant receptors." In addition to their mechanical properties, they are believed to be sensitive to many endogenous and exogenous chemical agents, such as histamine, nicotine, ammonia, and 5-Hydroxytryptamine

(5-HT), etc. Thus, RARs are believed to be polymodal (330). However, it is clear now the previously defined RAR category contains not only RARs but also other types of sensors. Many reflexes were attributed to RARs under the assumption that there are only three types of sensors in the airways. Observed phenomenon may be ascribed to RARs whenever SARs and C-fibers are ruled out. Thus, afferents such as HTARs and DARs were previously classified as RARs (403).

Deflation activated receptors Location and distribution. Since DARs were classified as a part of SARs and RARs, their general distribution along the airways should be the same as SARs and RARs but their exact location (in muscle or mucosal layers) has not been defined (403).

Afferent properties. Some mechanosensory units can be activated by lung deflation through activation of DARs. Mechanisms of DAR activation are unknown but must be related to the change in mechanical forces in the lung. DARs usually share axons with SARs and RARs in large animals, such as dogs, cats, and rabbits (403), as well as in small animals such as rats (158). They are inactive during eupenic breathing, but activated by forced lung deflation in large animals. Their conduction velocity should be in the same range of SARs and RARs. In small animals, discharge and conduction velocity of pure DARs were similar to SARs (23). In large animals, many of the DARs exhibit cardiac modulation during lung deflation (419). Matsumoto et al. reported that intravenous administration of veratridine, a sodium channel opener, stimulated DARs directly. Pretreatment with flecainide (a sodium channel blocker) attenuated the DAR response to lung deflation and blocked veratridine-induced stimulatory effects (251). Pure DARs are rare in large animals, but are common in small animals (23). For example, in spontaneously breathing rats, about 30% of mechanosensors are DARs (named as RARs by the investigators) (77).

HTARs Location and distribution. They have been identified in the large airways and in lung periphery. As with C-fibers, intrapulmonary HTARs are often located near the hilum. They have a much larger receptive field than mechanoreceptors, indicating multiple branching. This fits a net-work sensory ending formation, and their endings are located close to the lumen of the airway.

Afferent properties. Recently, airway sensors with a conduction velocity in the Aδ range (1.8-15 m/s) have been identified *in vivo* (236) and *in vitro* (386). At resting condition, many of them are inactive and those active ones discharged at less than 1 Hz on average. They are connected with myelinated afferents, and are neither RARs nor SARs. They share many characteristics with C-fibers, including chemosensitivity, a high mechanical threshold, and low discharge frequency even during high-pressure lung inflation. However, in rabbits they are not stimulated by phenyldiguanide, an agent that activates C-fibers in this species (361). These sensors are called high threshold Aδ receptors (HTARs). HTARs are stimulated by hypertonic saline, hydrogen peroxide, bradykinin, TNFα, and IL-1β (230, 236, 407). So far, there is no chemical agent has been identified to stimulate HTARs selectively. As stated, RARs are not well defined. Many afferent properties and reflex functions ascribed to RARs may belong to HTARs.

C-fibers Location and distribution. C-fibers are distributed from the trachea to the lung periphery (65, 67, 225), and represent ~80% of the vagal bronchopulmonary afferents (4, 179). They can be categorized into two major groups: pulmonary or bronchial C-fibers. Pulmonary C-fibers receive blood supply from pulmonary circulation and are often located in the lung periphery. Many of them respond to a stimulant with short latency, either delivered by right atrial injection to the pulmonary circulation or by aerosol to the airways. These sensors are believed to be located in the lung interstitium next to the pulmonary capillary. Thus, they are also called juxtacapillary or J receptors for short (283). C-fiber is a preferred term to J receptor, because many other types of sensors also have very short latency response after right atrial injection of a stimulatory or inhibitory agent. The "J receptor" described by Paintal appears to be a subgroup of pulmonary C-fibers. Bronchial C-fibers are perfused by systemic circulation via the bronchial artery and often located in large airways and superficially in the lumen. For simplicity, these two types of C-fibers are often reviewed together although there are some differences in their afferent properties and reflex effects (225) (also see page 19).

Afferent properties. Bronchopulmonary C-fibers discharge at low frequency with an irregular pattern under eupneic condition (299). C-fibers are chemosensitive. They are activated by a variety of endogenous and exogenous agents, such as hydrogen ions, adenosine, reactive oxygen species (ROS), capsaicin, and phenyldiguanide, and also activated by changes in osmolarity and temperature (see page 17). C-fibers are relatively insensitive to mechanical stimulation such as lung inflation, but respond to extreme hyperinflation (225). Therefore, they are not mechanically activated during normal breathing (190).

Summary Behaviors of chemosensors (C-fibers and HTARs) are similar, and mechanosensors (RARs and SARs) are also much alike, but chemical and mechanical sensors are very different. For example, RARs, similar to SARs are not sensitive to the chemical stimulation but vigorously stimulated by lung inflation (158). In that study 15% of the "RARs" were chemosensitive and could be HTARs. The chemosensors are stimulated by lidocaine transiently following an intraventricular bolus injection, whereas the mechanosensors are suppressed (229). During oleic acid induced acute lung injury, while both mechanosensors and chemosensors were activated, the sensitivity to lung inflation decreased in mechanosensors but increased in chemosensors (236, 237).

Cough receptors

Cough reflex is one of the most effective and rapid-responding defense functions that protect the lungs against inhaled

irritants and foreign substances. In conjunction with mucociliary system, cough can expel the irritant substances deposited in the airway lumen as well as the excessive airway secretion produced under abnormal conditions from the respiratory tract. Cough is a complex and exquisitely coordinated reflex action that generates a forceful expulsion of alveolar gas through the tracheobronchial tree. The neural pathways mediating the cough reflex consist of three major components: (i) cough receptors: specific sensory terminals located primarily, but not exclusively, in the respiratory tract; (ii) cough centers: neural circuits located in the brainstem and higher centers; and (iii) the respiratory pump: respiratory muscles in the abdominal wall, rib cage and diaphragm, and laryngeal adductor muscles. The afferent and efferent signals are mainly conducted in the vagus nerves and bulbospinal tracts, respectively.

Cough receptor is a functional definition for the sensors that initiate the cough reflex. It consists of multiple types of airway afferents with different sensory modality (e.g., chemical and mechanical), as characterized by different types of receptor proteins expressed on the neuronal membrane of the sensory endings. These cough receptors are located mainly in different regions of the respiratory tract, including larynx, tracheobronchial tree and alveoli; cough receptor located in other organ structures, such as esophagus (193) and tympanic membrane (394), have also been reported. A pioneering study by Widdicombe has reported a higher density of these receptors in the trachea and major bronchi, especially in the vicinity of carina, a strategic location in detecting inhaled irritants and protecting the lungs (384). In a series of recent studies, Canning and coworkers have identified a distinct subgroup of sensory nerves in guinea pig trachea and bronchi that elicits cough reflexes, and their function is not totally suppressed by general anesthesia (43, 44). These thin myelinated (Aδ) vagal afferent fibers innervating the extrapulmonary airways (trachea and larynx) exhibit exquisite sensitivity to punctuate mechanical stimulation and acid challenge, but are insensitive to capsaicin, and their activation by acid evokes coughing in a dose-dependent manner in anesthetized guinea pigs (42, 43) (Fig. 2). Furthermore, they are not activated by lung inflation, bronchoconstriction, or other autacoids known to stimulate RARs, and they conduct action potentials at a slower velocity ($<$8 m/s) than intrapulmonary RARs. The cell bodies of these cough receptors reside in the nodose ganglia. Interestingly, these receptors share some morphological features with SARs (42, 44, 256, 377, 403). Their sensory terminals can be identified by intravital labeling with the styryl dye FM2-10 or with antibodies against α3 subunit of the sodium pump, and are distributed in the airway mucosa, between the smooth muscle and the epithelium (42, 44, 256). In view of their distinct sensitivity to light punctuate stimulation, it seems conceivable that the activation of these receptors is also responsible for the airway irritation and coughs generated by the presence and/or movement of the mucus on the airway mucosa.

The evidence in support of an important role of bronchopulmonary C-fiber afferents in eliciting the cough reflex has also been extensively documented (see page 22). Stimulation of C-fiber afferents by inhalation of capsaicin or acid aerosol can consistently and reproducibly produce coughs in a dose-dependent manner in both healthy humans and in patients with airway inflammatory diseases (85, 88, 156, 188, 242, 395). A number of endogenous inflammatory mediators known to increase the C-fiber excitability, such as prostaglandin E_2 and bradykinin, also enhanced the cough sensitivity (56, 109). Furthermore, a substantial increase of C-fiber nerve profiles was found in the bronchial tissue of patients with chronic cough (133, 262). It is also possible that the tussive responses are elicited by indirect effects of the C-fiber stimulation; for example, a study by Joad and coworkers has demonstrated that stimulation of C-fibers in the airways can lead to the release of substance P (SP) and subsequent activation of RARs via the synthesis and release of nitric oxide which causes an increase in the interstitial fluid volume in the airways (182). Nevertheless, it seems undisputable that bronchopulmonary C-fiber afferents are involved, either directly or indirectly, in eliciting the cough reflex (41, 85, 188, 225, 390). In addition, indirect evidence suggests that afferent signals arising from bronchopulmonary C-fibers and mechanically sensitive cough receptors in the trachea may interact centrally at the brain stem and produce a synergistic effect on cough reflex (41).

Morphology of the sensory terminals

Having a better knowledge about the structure helps to understand how the sensor operates. However, our knowledge regarding sensor morphology and especially its relations to function is limited. Even SARs, the ones with most morphological information available, are characterized with little agreement on their structure, shape, location, and orientation (16, 91, 202, 398). The lack of morphological information further impedes our understanding of the sensory transduction mechanism.

Sensory innervation

The lung is innervated by the sympathetic and the vagus nerves. The vagal afferents are connected to nodose and jugular ganglia, and then to the NTS. The afferent fibers from the lung periphery run along the trachea in bundles. In the large airways, they issue a small cluster of axons or a single axon passing in the lamina propria (378). Their terminals may be connected to several receptor structures. Similar receptor structures have been identified in the reptile, rabbit, dog, cat, rat, mouse, and humans (403). Early studies with different staining techniques (methylene blue, silver impregnation, and zinc iodide) provide a fairly good account of airway innervation and receptor structures (403). However, they are descriptive and based on camera lucida drawings. With the advent of immunohistochemical staining techniques, Yamamoto, using antibodies against neurofilament protein (398) and calretinin (399), observed the sensory structure. With advances in immunohistochemistry with fluorescence

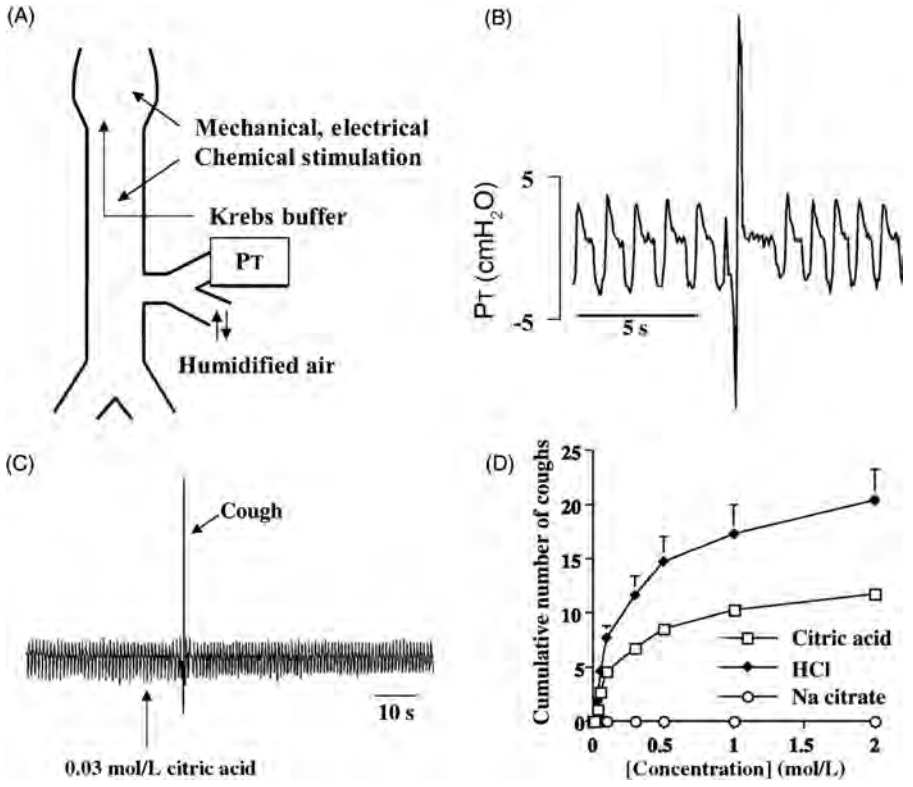

Figure 2 Sensitivity of tracheal cough receptors to acid and mechanical probing. (A) A schematic diagram of the preparation used to study cough in anesthetized guinea pigs. Pressure changes at the tracheal cannula (P_T) are used to monitor respiration and cough. The extrathoracic trachea was continuously perfused with Krebs bicarbonate buffer throughout the experiments. (B) A representative trace of coughing initiated by mechanically probing the tracheal mucosa. Cough is defined visually by the experimenter, and based on the magnitude of the inspiratory (appearing as a downward deflection in P_T) and expiratory (>500% of expiratory pressure during tidal breathing) efforts. (C) A representative trace of citric acid-evoked coughing. Citric acid (0.001-2 mol/L) was applied topically in 100 μL aliquots directly to the tracheal mucosa. Concentrations of citric acid ≥0.03 mol/L consistently evoked 1 to 2 coughs within seconds of application. (D) Cumulative coughs evoked by citric acid (n = 83), sodium citrate (n = 3), and HCl (n = 3). Note that the tussive effect of citric acid on coughing is mimicked by HCl but not by sodium citrate. [Modified, with permission, from references (42, 43).]

(Na^+/K^+-ATPase, for example), neural tracing, and confocal microscopy, the structures of sensory receptors can be examined in detail and evaluated objectively (1, 256, 377). FM2-10 is a hydrophilic dye that destains rapidly. Neurons fluoresce brightly during excitation. Thus, this activity-sensitive dye can be used for quantitatively assessing airway receptor responses to stimulating or inhibiting agents (255). Injecting anterograde tracing, fluorescent carbocyanine dye DiI, into the nodose ganglion, airway sensory structures have been demonstrated in rats (2, 368) and rabbits (408). Although confocal microscopy may provide an objective intuitive view of the receptor structures, electron microscopy is needed for delineating the structure at the subcellular level (91, 202).

Plant-like structure

In the airways, axons bifurcate to form receptor structures in different layers: epithelium, lamina propria, and smooth muscle (378). These structures are often innervated by myelinated fibers, which are demonstrated by their immunoreactivity with myelin basic protein (33). Although sensor morphologies vary in size, shape, orientation and levels of complexity, they have similar plant-like features. The main axon of a sensory receptor resembles the trunk of a tree. It gives off major branches, which further branch to form secondary and tertiary twigs. The terminal endings enlarge to form knob-like or leaf-like expansions. Receptor structures in peripheral airways (413) resemble those in central airways (212). However, the density is lower towards the periphery. Even in the trachea, the density is higher at the cranial end (16, 400). There are about 150 receptor structures in the full length of the guinea-pig trachea (16). A parent axon can give off branches forming several receptor structures that are buried in the muscle layer, which can be clearly demonstrated by confocal microscopy (377) or by double staining of receptor structures and airway smooth muscle (33). In dogs, several axons may form a large complex receptor structure (400). The size of the structure varies and is about 100 to 400 μm in length and 30 to 80 μm

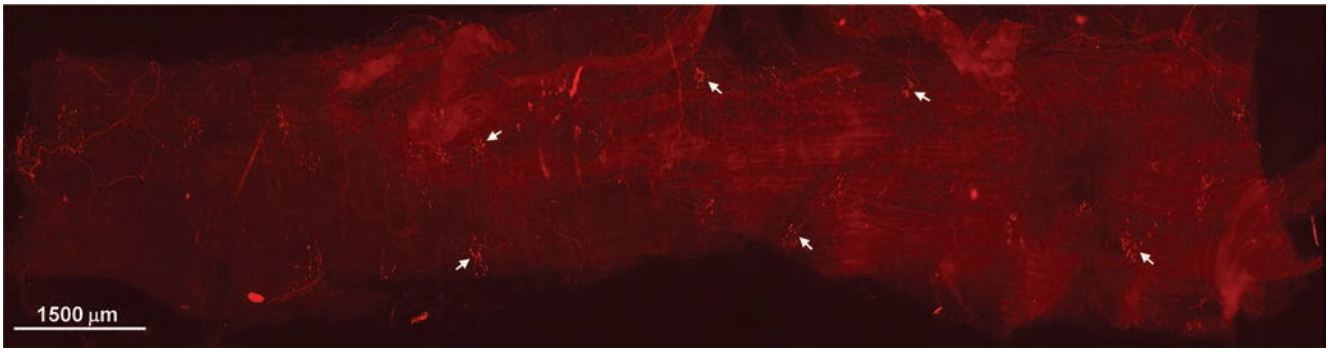

Figure 3 A macro-view of interrelationships of mechanoreceptors in one third of a rabbit tracheal smooth muscle ($15 \times 3.3 = 49.5$ mm^2). The tissue is immunohistochemically stained by antibodies for Na^+/K^+-ATPase to visualize the immunoreactive structures under a fluorescent microscope. Thirty one receptor structures are identified (some pointed by arrows). These structures are regularly spaced and have varied shapes and orientations. Most receptor structures have their long axis roughly running transversely. This figure not only shows detailed information of the "tree" (please see the online figure for enlarged views) but also global information for the "forest."

in width in dogs (398, 400). In rabbits, the structure extends 100 to 700, 60 to 200, and 20 to 70 μm in width, length, and depth, respectively (378). SARs have been described running in parallel with smooth muscles (16, 398). However, the sensory axis has never been clearly stated. In the peripheral airway, the direction of the smooth muscle fibers is irregular; therefore, a parallel relationship becomes impossible. If the knobs or leaves in sensory terminals are basic sensory devices, they can stretch in any direction no matter how the smooth muscle is oriented. Recently, SAR structure has been identified as plant-like by combining microdisection of the receptor and histochemical staining (413). However, the structure of RARs remains unknown, although they are believed to lie in the epithelium and deep layers of the airway wall (330).

Using montage, the sensors can be examined macroscopically to provide global information for the "forest" as well as detailed information of the "tree" (Fig. 3). These sensors are somewhat regularly spaced and have varied structures and orientations, although most sensors have their long axis roughly running transversely. On the other hand, at electron microscopic level, myelinated sensory endings, often accompanied by unmyelinated fibers, lose myelin and form ring-type end structures with close contact with collagen. The cytoplasm is filled with microtubules, mitochondria, glycogen, and osmiophilic bodies (91, 202, 400). Many of these features are shared by other types of mechanoreceptors, such as baroreceptors (403).

Network structure

Many vagal nerve endings in the trachea, bronchioles, and alveolar regions form network-like projections. In the airway varicose, nerve fibers lie in a plexus above the basal membrane (Fig. 4) (210). Deep in the lamina propria nerve trunks issue small nerve bundles supplying the basal layer. These nerve fibers travel between the epithelial cells to the apical epithelium, where they arborize into processes that terminate in enlarged varicosities (Fig. 4). Since receptive fields of C-fibers and HTARs cover large areas, these network endings could be C-fibers or HTARs. C-fibers, which account for 80% of all vagal pulmonary afferents, are believed to be immunoreactive with many neuropeptides, including SP and calcium gene related peptide (CGRP) (210, 343), forming network structures in the airways.

Neuroepithelial body

In the airway epithelium, there are numerous neuroendocrine cells. They can be singular or in clusters. Some of these cells are aggregated and richly innervated, named as neuroepithelial bodies (NEBs) (2, 70). The nerve fibers of NEBs emanate from the spinal cord and nodose ganglia (2, 368). NEBs take different shapes (Fig. 5) (34) and contain numerous bioactive substances and peptides [CGRP, SP, and protein gene product 9.5 (PGP 9.5) to name a few], which can be targeted by antibodies. Using the anterograde neural tracing technique with confocal microscopy, Van Lommel et al. observed that sensory nerve fibers were running in the airway walls and penetrated the epithelium, where they connected with NEBs (368). Subsequent electron microscopy revealed the ultrastructures of the NEBs as corpuscular cells containing dense cored secretory vesicles and contacted by mitochondria-rich nerve endings, which are connected with myelinated fibers (368). Myelinated-fiber innervation of NEBs has been vividly demonstrated by double staining of NEBs and myelin basic protein that labels myelin sheath (Fig. 5). NEBs are thought to be airway sensors (383). They are well defined structurally and have been reviewed recently (1, 71, 367). However, nothing is known about their afferent discharge patterns. Many functions have been proposed for NEBs (344), such as oxygen sensors (70), mechanosensors (35, 267), and chemosensors (344, 402). It has long been suspected that the NEB is one known type of airway sensors (368, 402). From a morphological standpoint, NEBs resemble the receptor structure of chemosensors, but not that of mechanosensors. In addition, mechanosensors (SARs and RARs) are abundant in the trachea, whereas few NEBs can be found in the trachea. When fluorescent dye is injected into the nodose ganglia, the

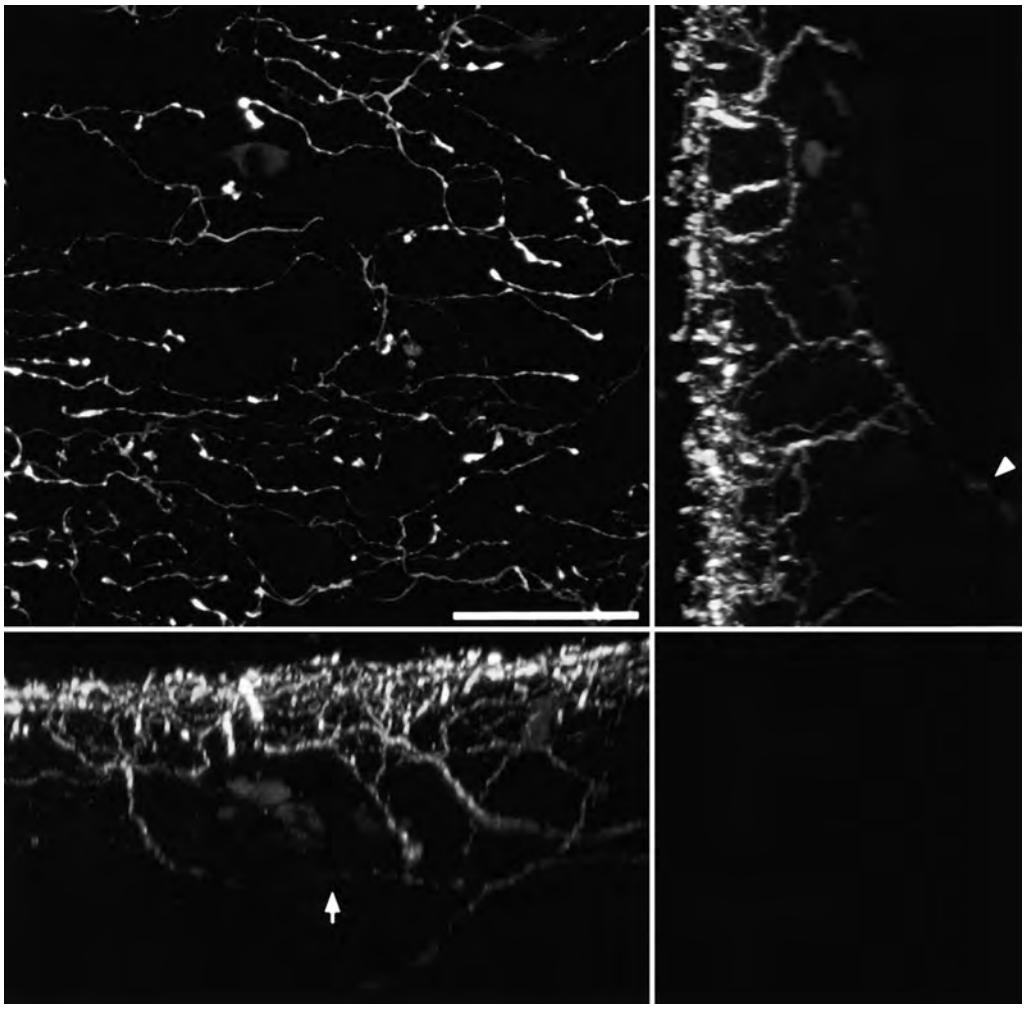

Figure 4 Confocal microscopic views of a network structure of SP-IR nerve fibers in bronchial mucosa of a pig (*bar* = 50 μm). Large panel, a lumenal projection, shows nerve terminals in the mucosa have swollen varicosities. Right and bottom panels are cross-sectional projections, where nerves in the epithelium converge into bundles as they enter the lamina propria. Some bundles run longitudinally along the airway below the epithelium (arrow) and the others penetrate deeper (arrowhead). [Modified, with permission, from reference (210).]

structures of NEBs (368, 408) are quite different from SARs (403). The two different entities demonstrated by neural tracing results were confirmed with histochemical staining (415). Na^+/K^+-ATPase positive SAR structures were not observed in NEBs. Tissue blocks containing SARs showed a receptor structure but not NEB structure after proper staining (415). Since SARs are neither SP-immunoreactive (IR) nor CGRP-IR, it is unlikely that NEBs are connected with mechanosensors. If the NEB does participate in the mechanotransduction of SARs, it must play a modulatory role. NEBs may connect with chemosensors, such as HTARs and C-fibers, or sympathetic afferents, because NEBs possess the bioactive substances that can activate chemosensors (402). Since myelinated afferents are believed to innervate the NEB and since HTARs are myelinated and many of them are located in intrathoracic airways, HTARs seem to be the best candidates for innervating NEBs.

Transduction mechanisms

Sensory receptors are activated in response to changes in mechanical or chemical conditions in the lung. Transduction mechanisms occur in tissue (intracellular), cellular, and molecule levels.

Mechanosensitive afferents

Stimuli that activate mechanosensors Airway mechanosensors are activated by changes in mechanical properties and conditions of the airways and lung, such as lung deflation and inflation. Whether different stimuli (lung inflation and deflation) can activate a given sensor is still being debated. Conventionally, it is believed that a SAR or RAR can respond to both inflation and deflation (one-sensor theory). Such a theory has been accepted without testing its validity. A sensory unit capable of responding to both lung

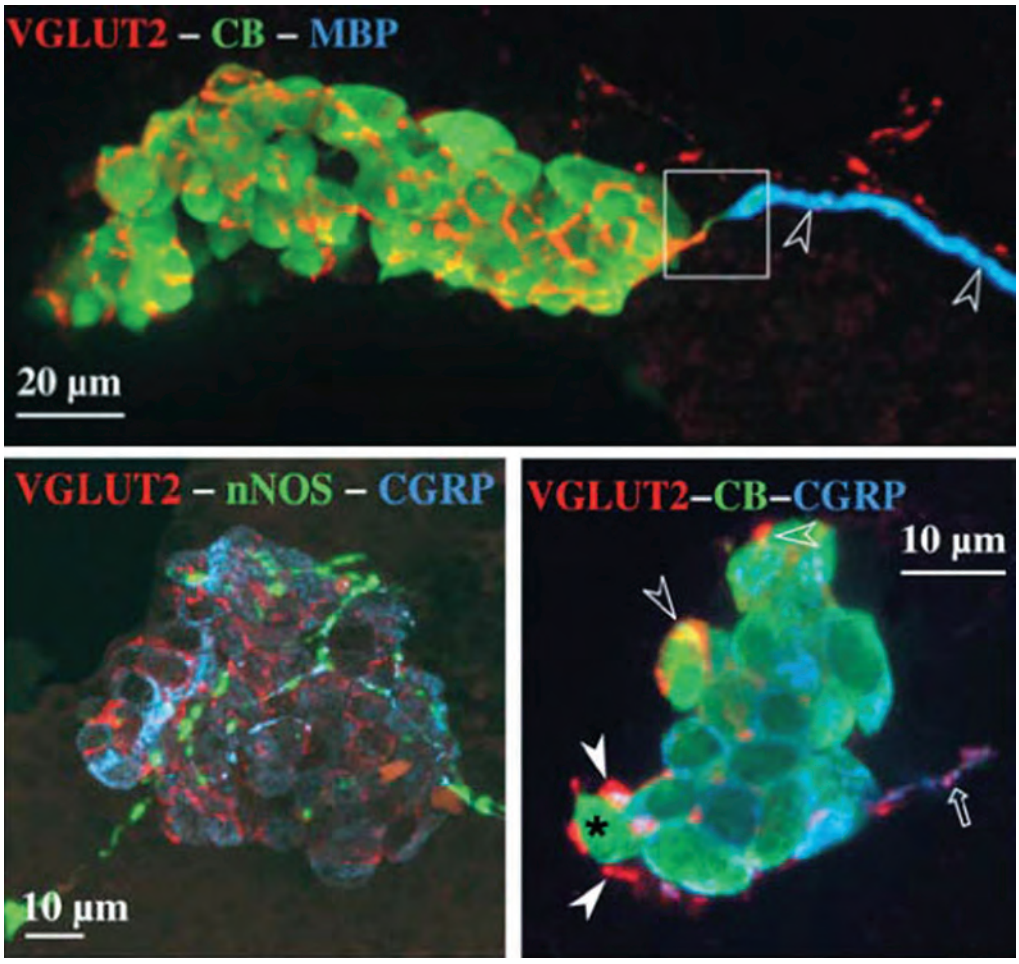

Figure 5 NEBs are richly innervated by different types of nerve fibers. They are taking different shapes and immune reactive to a variety of antibodies. They can be cylindrical (top), ball shaped (bottom left), or irregular (bottom right). Top figure, immunocytochemical triple staining for VGLUT2 (vesicular glutamate transporter 2; red), CB (calbindin D28 k; green) and MBP (myelin basic protein; blue), shows that a NEB is contacted by a CB-IR vagal nodose sensory fiber that is surrounded by myelin sheath (MBP-IR; open arrowheads). VGLUT2 IR is seen in nerve terminals between the NEB cells. Bottom left, triple stained for VGLUT2 (red), CGRP (blue), and nNOS (neuronal nitric oxide synthase; green), shows a NEB receiving nNOS-IR nerve fibers. Bottom right, triple stained for VGLUT2 (red), CGRP (blue), and CB (green), shows that a NEB is contacted by a CGRP+/CB+/VGLUT2+ spinal sensory fiber (open arrow) and red nerve fibers (arrowheads) surround a NEB cell (asterisk). [Modified, with performance, from reference (34).]

inflation and deflation has been shown to respond to deflation only after the inflation receptive field is blocked by local anesthetics, and vice versa (see page 11). This demonstrates that a sensory unit contains more than one sensor (multiple-sensor theory). Thus, responses to different stimuli may come from different sensors. While how mechanosensors are exactly activated is not known, they must be activated by deformation of the tissue where the sensors are located. Mechanosensors are generally insensitive to chemicals, except those can alter lung mechanics. For example, ACh, 5-HT, and histamines may cause airway constriction, and stimulate mechanosensors by secondary effects (65). In addition, any chemical agent which modifies the mechanical-sensitive ion channels can alter the mechanosensory activity (see page 26). Recently, it was reported that mechanosensors may express TRPV1 receptors and become chemosensitive following chronic airway inflammation (418).

Cellular mechanism of activation Signal transduction mechanisms, that is, how the information of the stimulus is transformed into an action potential train transmitted via the afferent axon, have not been directly studied in airway mechanosensors. An operative model has been proposed (403). In brief, information from numerous single stretch-activated channels is integrated through temporal and spatial summations, producing a generator potential (GP) that encodes overall information for the sensor in the form of action potentials (Fig. 6). Currently, there is no information regarding what mechanosensitive channels are responsible for signal transduction in airway mechanosensors. However,

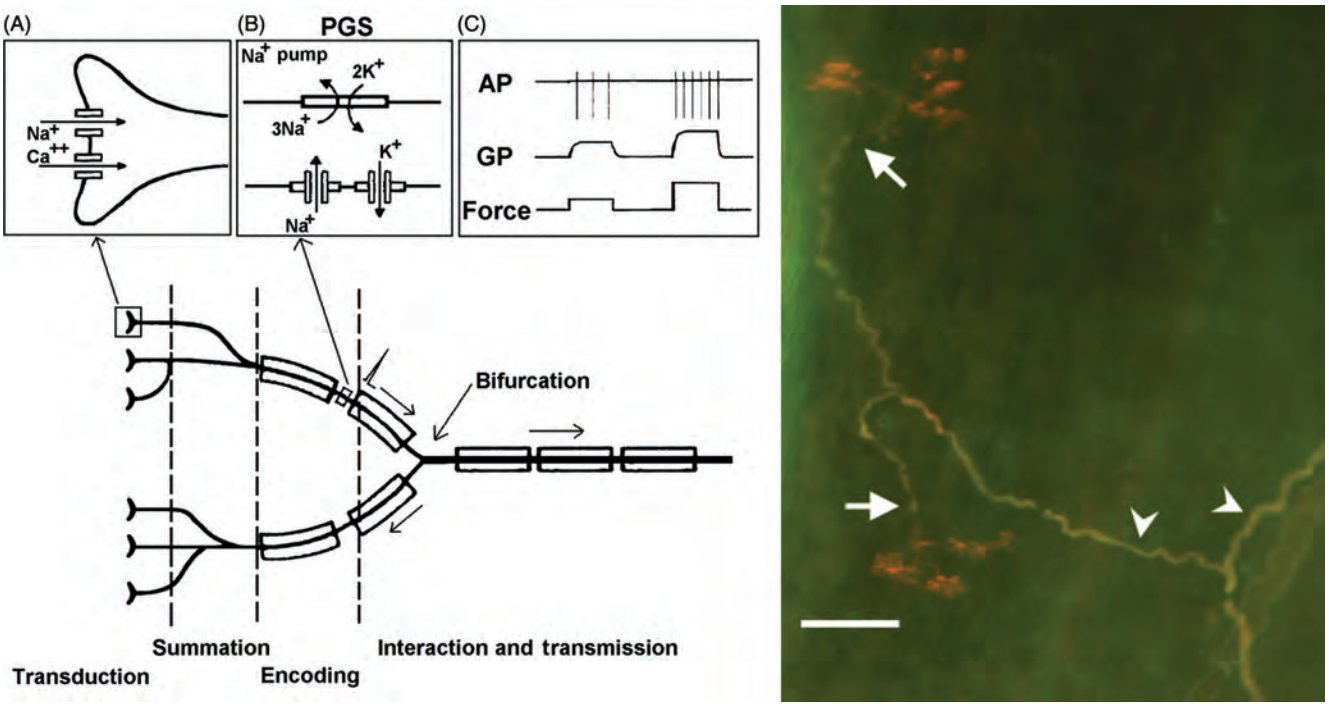

Figure 6 A schematic model of a sensory unit with two encoders (left) and a double staining approach to illustrate a sensory unit identified in a rabbit medium sized airway (700 μm; right). The sensory unit's functional structures can be divided into four regions: transduction, summation, encoding, and interaction/transmission. Mechanoelectrical transduction occurs in the transduction region. Mechanical force opens stretch-activated cation channels, causing cations influx and membrane depolarization in the terminal (inset A). These local depolarizations summate temporally and spatially in the summation region. Summation determines the size of generator potential (GP) at the potential generating site (PGS) in the encoding region (inset B). When the GP reaches threshold, action potentials (AP) are produced by opening and closing of Na^+ and K^+ channels; the ion disturbance is restored by a Na^+ pump (inset B). AP discharge at the PGS is proportional to the GP, which, in turn, is directly related to the force acting on the receptor terminals (inset C). At the interaction and transmission region, AP reaching the bifurcation will propagate antidromically toward the other encoder for interaction, and centrally for further information processing (see arrows). The sensory unit integrates information by an amplitude modulating mechanism before encoding, and by a frequency modulating mechanism after encoding. At the right the sensory structures are labeled by Na^+/K^+-ATPase (red), and myelin basic protein (MBP; green) antibodies; yellow parts show costaining (red+green). The parent axon of the sensory unit is running from the bottom up at the right side. It gives off two branches (left and right) indicated by an arrow head, respectively. The left branch further divides into two. Clearly, the axon demyelinated at the junction (pointed by the arrow) to the sensory device (encoder, pure red portions without costain with MBP), where action potentials are generated. The white bar is 50 μm in length. Please note that two encoders in the unit can be homogenous (both encoders being SAR or RAR) or heterogeneous (that is one encoder is SAR and the other is RAR or DAR; or one encoder is RAR and the other is DAR). [Left figure is adapted, with permission, from reference (403).]

stretch-activated cation channels are present in other organ systems. The degenerin/epithelial sodium channel is suggested to operate in baroreceptors (90). Activation of this channel results in an influx of cations. The ion concentration across the sensory membrane is restored by the sodium pump or Na^+/K^+-ATPase, which is located at the sensory terminals and the potential generating site. Since the sodium pump is electrogenic (357), inhibition of the pump may prevent ion restoration and sustain the GP, producing action potentials (389). Opening of voltage-dependent sodium channels accounts for the rapid rise of action potentials. For example, SARs are stimulated by veratrine (Na^+ channel opener) and blocked by TTX (blocker for TTX-sensitive Na^+ channels) (253). The 4-AP sensitive K^+ channels, as well as the sodium pump are responsible for the repolarization (51, 257).

Concept of sensory unit The afferent activity recorded from the vagus nerve is not from a single sensor, but from a single sensory unit (Fig. 6). A sensor or receptor is an encoder that may independently respond to an adequate stimulus to generate action potentials. A mechanosensory unit is a functional unit that possesses multiple sensors, which can be homogenous or heterogeneous (403). These sensors can be activated by inflation (RARs and SARs) or deflation (DARs). A significant amount of information is integrated at the sensor and unit levels. In a sensor, analog signals are encoded in GP (amplitude modulation), which is transformed into action potentials that are encoded as frequency (frequency modulation). These action potentials from different sensors interact through a competitive selection mechanism to give the final output to the CNS. Thus, the sensory unit is not only a transducer, but also a processor (403).

Anecdotic reports suggest the existence of multiple encoders in airway mechanosensory units. In 1933, Adrian showed that activity from a SAR sensory unit shifts from regular and high frequency to irregular and low frequency during lung hyperinflation (3), dubbed the Wendensky effect. The phenomenon was attributed to a damaged sensory

apparatus. In 1989, studying airway SARs, Ogilvie and co-workers observed two sensory units that had abrupt changes in the variability of discharge during lung inflation. They suggested that the sensory units may have multiple sensors and the abrupt change was due to a pace maker switch (277). The importance of this phenomenon has never been emphasized because such sensory units account for only a small fraction of the sensor population. However, a recent report shows that about 25% of SAR units abruptly shifted their discharging frequency to a lower level during an 8-s lung inflation at 30 cmH_2O. This shift (pace maker switch) is due to deactivation of the high discharging sensor, leaving the low discharging one active (140). The deactivation probably results from a shortage of ATP supply. Since deactivation is directly related to the pressure and duration of lung inflation, higher and longer lung inflation will reveal more units with multiple sensor behavior. Thus, a significant portion, if not all, of the SAR units contain multiple sensors.

In studying RAR units in dogs, Sant'Ambrogio et al. (328) demonstrated that after removal of a superficially located receptive field (responding to touching), the units could still respond to lung inflation and deflation, indicating that a single sensory unit may respond to two different stimulating modes. Fig. 7 illustrates a sensory unit with two receptive fields and demonstrates that each field possesses at least one sensor (or encoder). Blocking a field with local anesthetics decreases total activity, but not peak activity. This suggests that the blocked field does not contribute to the peak frequency, that is, the peak activity comes only from the remaining receptive field. Thus, a receptive field can produce its own GP to generate action potentials, that is, contains its own sensor. An unit may contain two to four receptive fields, which are separated from a few mm up to 1 cm (414), or may even be in different lung lobes (403). Multiple receptive fields are found in a single sensory unit in the gastrointestinal tract (24, 417), and in other visceral systems innervated by sympathetic afferents (18). Multiple sensors are also found in a mechanosensory unit in the skin (165), tendon organs (114), and muscle spindles (47). In this case, at a given time domain, the sensor having the highest firing rate serves as a pacemaker and determines the unit discharge frequency (132). The pacemaker can switch from one sensor to another in a respiratory cycle. For example, in Fig. 7, the high-threshold sensor dominates in lung inflation phase, but the low-threshold sensor dominates in deflation phase. When two sensors have similar frequencies, because of variability in action potential occurrence, either sensor can be the pacemaker. Such sensor interaction follows probabilistic mixing (92), which improves regularity and increases the firing frequency. A sensory unit possessing multiple sensors can extend operating ranges and sensory modes. Thus, they may respond to different stimuli such as inflation and deflation. If the afferent is connected to only RARs or only SARs, the unit will be a typical RAR or SAR. If the afferent is connected to both RARs and SARs, it will present as an RAR-like SAR (405). Yet, if a unit is connected also to DARs, it can respond to lung deflation. This may explain the varied behavior seen

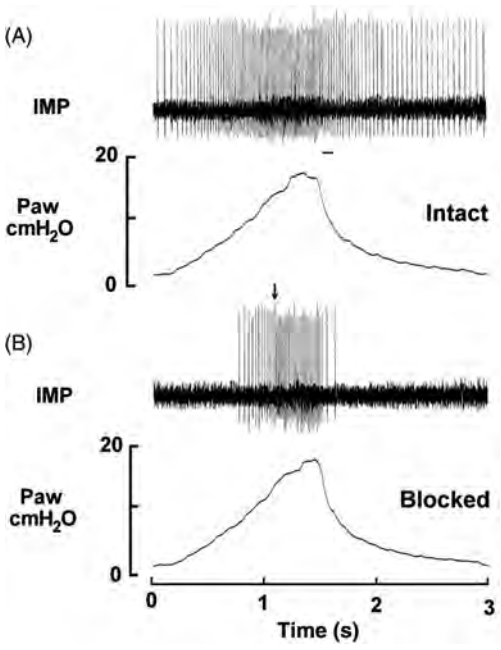

Figure 7 Activity of an SAR unit with two encoders: one gives high discharge frequency with a high threshold (encoder H), whereas the other produces low discharge frequency with a low threshold (encoder L). A is control, where the unit activity is from both encoders; B is after blocking encoder L with lidocaine. Thus, the unit activity is from encoder H only. The bar in A indicates where unit activity switches from encoder H to encoder L. The maximal frequency of encoder L is somewhere between the frequency at the bar and at the peak (the maximal frequency of encoder H). The arrow in B indicates where discharge of encoder H is about to exceed that of encoder L. Drawing two vertical lines crossing figure A at the arrow in B and at the beginning of the bar in A divides the time into three sections. The activity during deflation (first and third sections) is determined by encoder L, whereas the activity during inflation (second section) is determined by encoder H. Thus, the pacemaker switches back-and-forth between the two encoders within a ventilator cycle. Please see Fig. 6 for a schematic illustration for interaction of two encoders in a unit. IMP, impulses; Paw, airway pressure. [Adapted, with permission, from reference (414).]

in different sensory units. Both RARs and SARs sharing an axon are found in the muscle spindle (48, 174). Thus, viewing mechanosensory units as one group (instead of many groups) with a spectrum of behaviors is a reasonable approach (405).

Signals encoded for multiple variables being transmitted by a single afferent fiber raise the question: "How does the CNS decode?" There is no known answer to this. However, there are many potential mechanisms that may achieve this. For example, a sensory neuron may synapse on different NTS regions with different types of decoders to extract different information. Alternatively, different types of decoders may coexist in a particular region, each may extract a specifically encoded information (403).

Chemosensitive afferents

Chemical stimuli of bronchopulmonary afferents

In general, there are two major categories of chemical stimulants that bronchopulmonary afferents are susceptible to: inhaled irritants (e.g., cigarette smoke, sulfur dioxide,

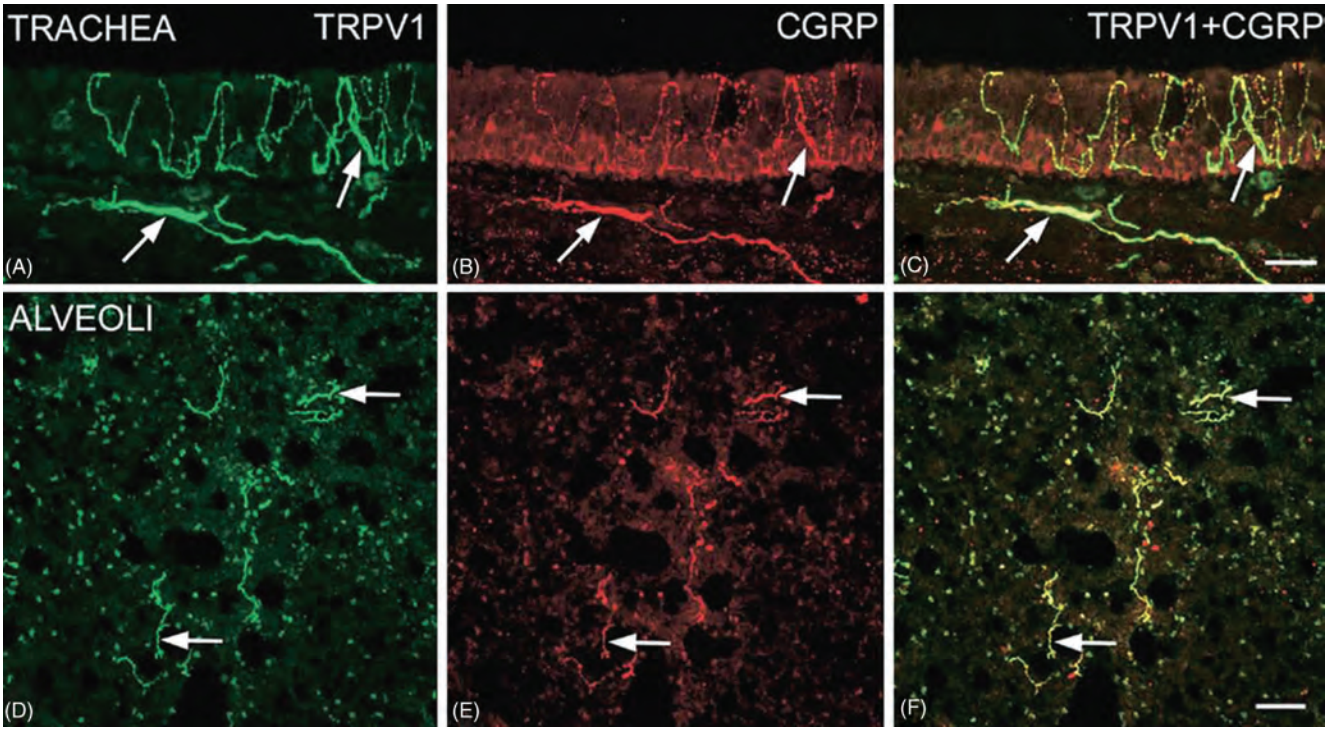

Figure 8 Coexpression of transient receptor potential vanilloid type 1 channel (TRPV1) and calcitonin gene-related peptide (CGRP) in airway sensory nerves. Confocal images showing TRPV1 (A, D) and CGRP (B, E)-immunoreactive axons in the guinea-pig trachea (A-C) and alveoli (D-F). Merged images illustrating co-localization of TRPV1 and CGRP are also shown (C, F). In the epithelium of the trachea (A-C), a fine network of coexisting TRPV1- and CGRP-immunoreactive axons is visible, derived from the subepithelial plexus. Arrows indicate double-labeled axons. In the lung, TRPV1-immunoreactive axons (arrows) are also visible within parenchymal lung tissue (D), and extensively colocalize with CGRP (E, F). Scale bars are 20 μm (A-C) and 100 μm (D-F). [Modified, with permission, from reference (381).]

etc.) and endogenous chemical substances (e.g., lactic acid, ATP, etc.). Indeed, the chemosensitivity of pulmonary afferents is not only important in protecting the lung and body from potential health-hazardous effects caused by air-borne chemical irritants, but also in regulating airway function under various pathophysiological conditions. The majority of these chemosensitive afferents are nonmyelinated (C-) fibers, though a small subgroup of myelinated (Aδ) afferents can be also activated by certain chemical stimulants (43, 158, 385). Immunohistochemical studies clearly illustrated the presence of C-fiber sensory endings in the lung parenchyma and in the mucosa of all sizes of airways in various species including humans (195, 381). These nerve endings display extensive axonal arborization that either extends into the space between epithelial cells or forms network-like plexus immediately beneath the basement membrane of airway epithelium (Fig. 8) (2, 17, 381). The superficial locations of these nerve endings in the airway lumen, and their distinct sensitivity to chemicals and ability in eliciting protective reflexes suggest an important role of these afferents in regulating the airway responses to inhaled irritants.

These chemosensitive afferents in the lung can be further divided into a number of sub-groups based upon their sensitivities to specific chemical agents, but further division serves little purpose because of the wide overlaps of sensory modality and reflex function among these different subgroups.

Cellular mechanisms of activation Similar to the sensory nerves innervating other parts of the body, the chemosensitive properties of the lung afferents are characterized by the presence of specific protein molecules (receptors) on the membrane of nerve terminals. Chemical agents activate these receptors and evoke action potentials in these afferents. In general, these receptors can be divided into two major categories: ionotropic receptor (ligand-gated ion channel) and metabotropic receptor (G-protein-linked receptor). Recent studies have demonstrated that several metabotropic receptors such as prostanoid receptors and bradykinin receptors are coexpressed in the same pulmonary sensory neurons (134, 203, 207, 252). These G-protein-coupled receptors play an important part in regulating the sensory excitability, particularly under various pathophysiological conditions (see page 26).

A number of well-characterized ligand-gated ion channels such as TRPV receptors, TRP ankyrin type 1 receptor (TRPA1), acid-sensing ion channels (ASICs), purinoceptor P2X2 and P2X3, and nicotinic acetylcholine receptors (nAChRs) are expressed in pulmonary sensory neurons (25, 26, 137, 206, 219, 265, 268). Among them, TRPV1 has drawn special interest and extensive investigation after it was first cloned in 1997 (50) because TRPV1 is generally considered as a selective biomarker for identifying C-fiber afferents (158). TRPV1 is a tetrameric membrane protein with

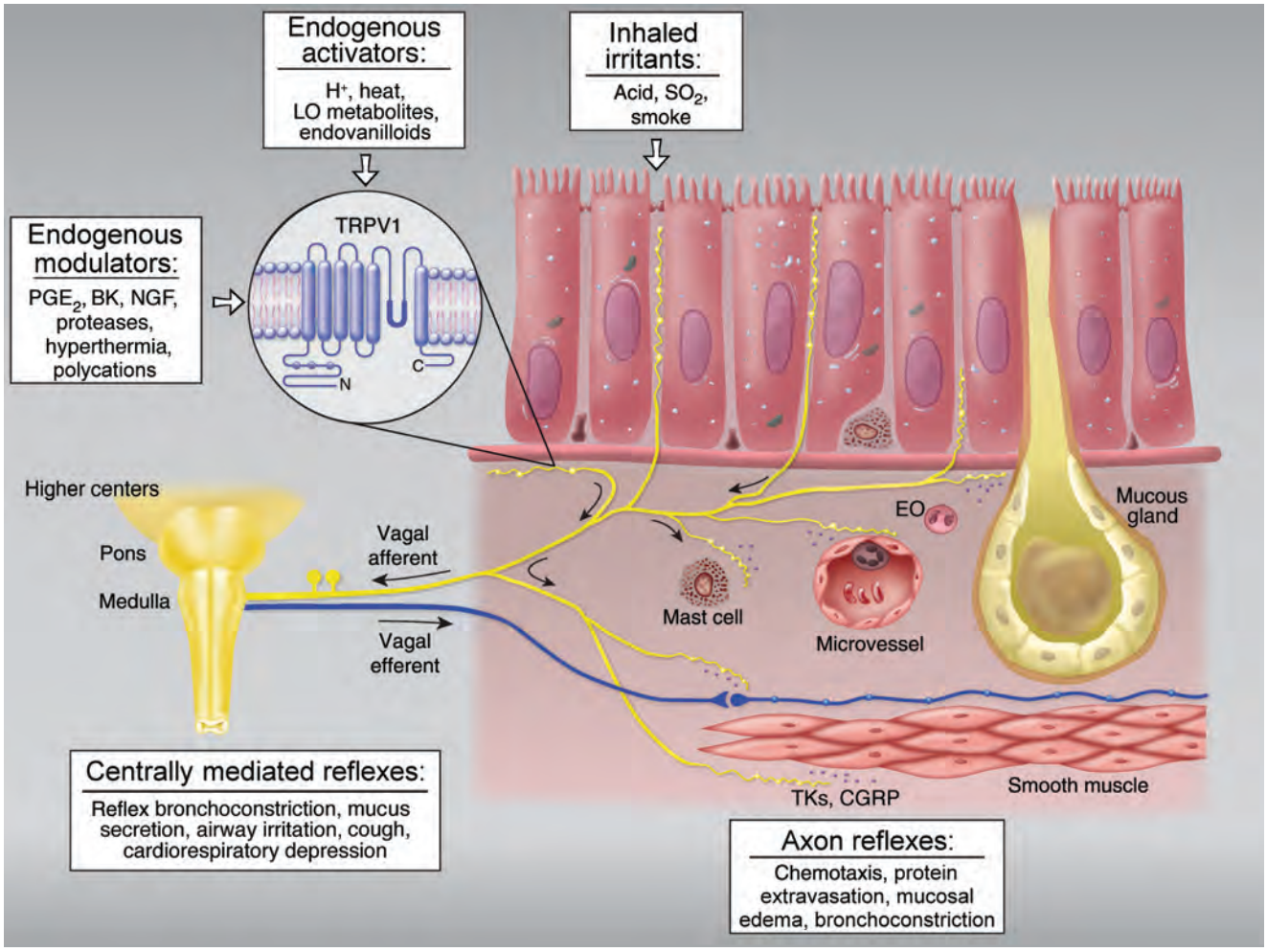

Figure 9 Schematic illustration of the function of TRPV1-expressing sensory nerves and their interaction with other cell types in airway mucosa. SO_2, sulfur dioxide; EO, eosinophil; LO, lipoxygenase; PGE_2, prostaglandin E_2; BK, bradykinin; NGF, nerve growth factor; TKs, tachykinins; CGRP, calcitonin gene-related peptide. See text for details. [Modified, with permission, from reference (220).]

four identical subunits, each containing six transmembrane-spaning domains, which form a nonselective cation channel with a high permeability to Ca^{2+} (50, 272). TRPV1 is a polymodal transducer that can be activated by a number of endogenous chemical substances and physiological factors, including hydrogen ion, bradykinin, certain lipoxygenase metabolites, and hyperthermia (121, 180). TRPV1-expressing nerve endings are extensively found in the airway mucosa as well as in the deeper layer of airway tissue (381) (Fig. 8). When they are activated either by inhaled irritants or endogenous TRPV1 activators, centrally mediated reflex responses are elicited, which include bronchoconstriction and mucus hypersecretion via the cholinergic pathway, accompanied by the sensation of airway irritation and urge to cough (Fig. 9). Activation of TRPV1 also triggers calcium influx and release of tachykinins and CGRP from the sensory terminals. These sensory neuropeptides can act on a number of effector cells in the respiratory tract (e.g., smooth muscles, cholinergic ganglia, mucous glands, and immune cells), and elicit the local "axon reflexes" such as bronchoconstriction, protein extravasation and inflammatory cell chemotaxis (80, 220) (Fig. 9). Furthermore, the sensitivity of TRPV1 can be enhanced by a number of endogenous inflammatory mediators such as prostaglandin E_2 and bradykinin (121, 180, 207) (Fig. 9). Indeed, increasing evidence suggests that the TRPV1 channel plays an important role in the manifestation of various symptoms of airway hypersensitivity, a common pathophysiological feature in patients with airway inflammatory diseases.

TRPA1, another member of the TRP family of ion channels expressed in a subset of bronchopulmonary TRPV1-expressing C-fibers (265), can also be activated by a number of chemical irritants (e.g., acrolein, toluene diisocyanate, etc.), endogenous inflammatory mediators (e.g., bradykinin, etc.), and ROS (12, 25, 238, 355). TRPA1 may be also involved in triggering the adverse pulmonary effects induced by acute exposure to ozone and other oxidants (26, 354), and in eliciting the cough reflex and development of airway hyperreactivity induced by active sensitization with allergen (13, 27,

38, 122). Some of these effects on airway reflex responses, such as cough, bronchoconstriction, respiratory inhibition, and mucous hypersecretion (26, 27, 354), appear to be similar to those mediated by activation of TRPV1 (67, 225). Despite of several attempts made in recent studies, the relative contributions of TRPV1 and TRPA1 to the overall defense mechanism involving airway sensory nerves are yet not clearly defined, and a potential interaction between these two transducers via certain signaling molecule(s) and second messenger(s) at the cellular level remains to be explored (7).

Hydrogen ion has been well recognized as a chemical irritant in the airways. The concentration of hydrogen ion in the form of lactic acid increases in the extracellular fluid of inflamed and ischemic tissues. Thus, in patients during asthmatic exacerbation, the pH of the airway vapor condensate of exhaled gas is reduced to 5.23, as compared to 7.65 in healthy individuals; this abnormally low airway pH returns to normal after anti-inflammatory therapy (175, 196), suggesting the tissue inflammation as the origin of airway acidosis. The evidence of acid stimulation of airway C-fiber afferents was first reported by Lou et al. (240) in isolated guinea pig lungs. The electrophysiological recording experiment showed that C-fibers innervating the trachea were stimulated when the isolated guinea pig airway was perfused with acidic buffer at pH of 5.0 (111). Furthermore, lactic acid, produced endogenously in large quantity during anaerobic tissue metabolism, stimulates pulmonary C-fibers in a dose-dependent manner (pulmonary venous blood pH: 7.09-7.29) in anesthetized rats, and hydrogen ions are primarily responsible for the action (163, 224). A direct stimulatory effect of hydrogen ions on TRPV1s was recently demonstrated in pulmonary sensory neurons isolated from rat nodose and jugular ganglia (136, 194). The current evoked by lowering the pH of extracellular solution to 7.0 consisted of only a transient, rapidly inactivating component with small amplitude, which increased in amplitude when the H^+ concentration was elevated. In addition, a slow, sustained inward current began to emerge when pH was lowered to below 6.5 (136, 194). The transient component was dose-dependently inhibited by amiloride, a common blocker of ASIC channels (373, 374), whereas the sustained component was almost completely abolished by capsazepine, a selective TRPV1 antagonist, indicating a critical involvement of TRPV1 in the acid-evoked current in these neurons (Fig. 10) (136). Sulfur dioxide, a potent irritant gas, activates pulmonary C-fibers and a small subset of RARs (158). It is conceivable that its stimulatory effect is caused by hydrogen ions in the sulfuric acid resulting from hydration of SO_2 in the airway lining fluid (146), though the direct evidence has not yet been established.

Previous investigators have suggested the existence of "CO_2 sensors" in the pulmonary circulation, and proposed an important role of these receptors in detecting the increase in venous CO_2 flux in the lung, and in regulating ventilation and maintaining arterial CO_2 homeostasis during exercise (289, 379, 380). However, studies by others indicated that lung vagal

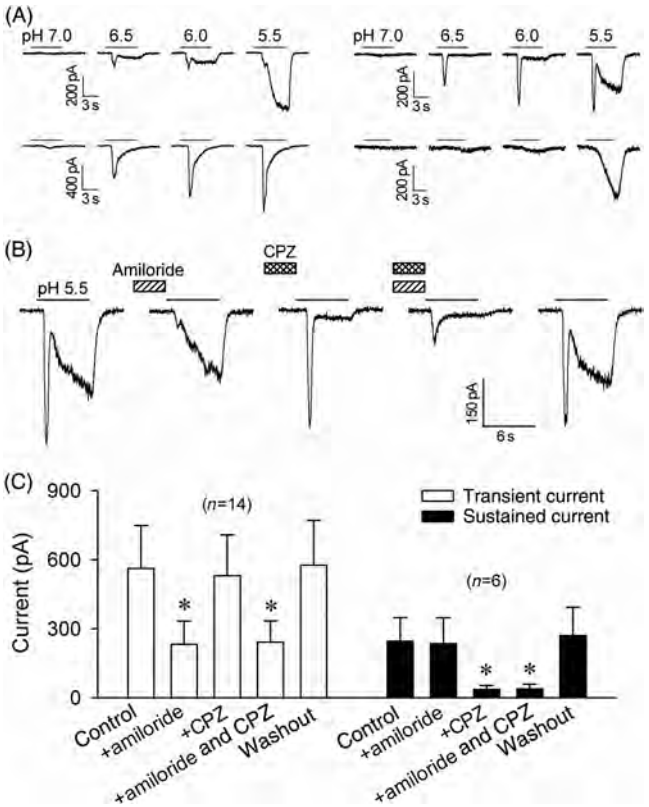

Figure 10 Acid-evoked whole cell inward currents and inhibition by amiloride and capsazepine in rat vagal pulmonary sensory neurons. (A) Low pH with increasing proton concentrations are applied for 6 s (as indicated by the horizontal bars) to four different neurons. Note distinct pH sensitivities and different phenotypes of inward currents in response to acidic challenges. (B) Effect of 2-min pretreatments with amiloride (100 μmol/L) and capsazepine (CPZ; 10 μmol/L) on acid (pH 5.5)-evoked inward currents. (C) Acid (pH 6.5-5.5)-evoked both transient and sustaining components are inhibited by amiloride and CPZ. $^*P < 0.05$ as compared with the corresponding control. [Modified, with permission, from reference (136).]

afferents are not required for regulating normal ventilatory response to exercise (21, 58, 98, 104, 130). An in-depth discussion of this subject was presented in a recent review (106). In addition, when the conduction of myelinated fibers in both vagus nerves was selectively blocked by either differential cooling (290) or anodal hyperpolarization (321), the increase in respiratory rate during the hypercapnic challenge persisted, further suggesting an important role of vagal bronchopulmonary C-fibers in the hyperpneic response to CO_2. In view of the distinct sensitivity of pulmonary C-fibers to hydrogen ion as described above (136, 163, 224), it seems plausible that an increase in either the venous CO_2 flux during exercise or the alveolar CO_2 during hypercapnic challenge may lead to a decrease in the pulmonary interstitial pH, which can then activate the pulmonary C-fibers. However, the follow-up study performed by the same group of investigators could not find any conclusive evidence indicating that vagal bronchopulmonary C-fibers act as CO_2 sensors in the healthy lung under resting conditions (234). Whether the weak response

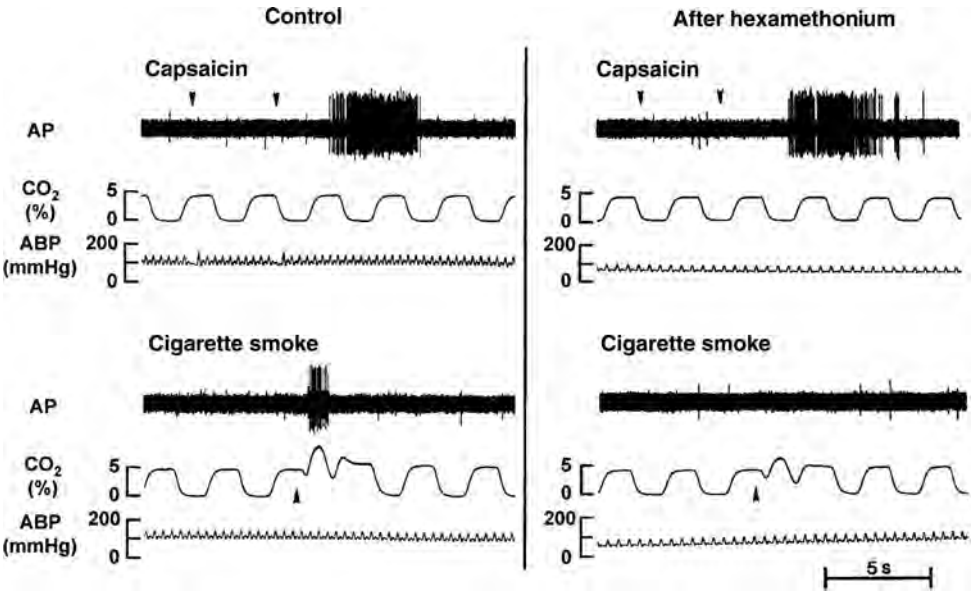

Figure 11 Specificity of blocking effect of hexamethonium on pulmonary C-fiber response to cigarette smoke. The location of the sensory terminal was identified in the right cardiac lobe of an anesthetized and open-chest dog. Left: control responses; right: responses 5 min after hexamethonium (0.8 mg/kg, i.v.). Above: capsaicin (2 μg/kg) injected into catheter at first arrow and flushed into right atrium at second. Below: 120 mL high-nicotine cigarette smoke delivered to lungs in a single ventilatory cycle; note sudden increase in inspiratory CO_2 concentration. Arrow, whose position has been adjusted to correct for time lag of CO_2 analyzer, indicates arrival of smoke in airways. Note that hexamethonium decreased mean arterial blood pressure by about 45 mmHg. Interval of 20 min elapsed between injection of capsaicin and inhalation of smoke. AP, action potentials; CO_2, CO_2 concentration in trachea; ABP, arterial blood pressure. [Modified, with permission, from reference (222).]

of pulmonary C-fiber afferents to venous CO_2 loading is potentiated by concomitant increases in body temperature (215, 317) and/or pulmonary interstitial fluid pressure (285) occurring during severe exercise remains to be determined (see page 17).

Cigarette smoke causes airway irritation and coughing, and is undoubtedly one of the most common inhaled irritants to the human respiratory tract. Inhalation of a single breath of cigarette smoke can stimulate both C-fibers and RARs in the lungs in various animal species (200, 222). More importantly, this stimulatory effect is primarily caused by nicotine contained in the cigarette smoke (201, 222) (Fig. 11). The studies conducted in healthy nonsmokers further confirmed that the irritant effect of inhaled cigarette smoke in human respiratory tract was indeed generated by the action of nicotine (218). Furthermore, in isolated pulmonary sensory neurons, nicotine also evoked a pronounced and abrupt calcium influx in approximately one third of the pulmonary sensory neurons tested (397). The irritant effect of nicotine is believed to result from an activation of neuronal nicotinic acetylcholine receptors (NnAChRs) expressed on the sensory terminals located in the airway mucosa because both the cough reflex in humans and responses recorded in sensory nerves and isolated neurons can be prevented by a pretreatment with hexamethonium (Fig. 11). In addition, patch-clamp recordings have demonstrated that both nicotine and acetylcholine depolarized the membrane potential, generated action potentials and evoked inward current in a concentration-dependent manner in these neurons. The expression of mRNA encoding for the nAChR subunits α4-7 and β2-4 in pulmonary sensory neurons as demonstrated by the RT-PCR analysis lent further support to this conclusion (137).

Despite that a distinct role of nicotine in evoking airway irritation, cough, and stimulatory effect on vagal airway afferents in humans, dogs, and guinea pigs (164, 218, 222), the relatively low or no sensitivity of vagal afferents to nicotine was found by the same investigators in rats (223). In anesthetized rats, activations of the TRPA1 receptors by endogenous cyclooxygenase (COX) metabolites and the P2X purinoceptors by ATP are primarily responsible for the stimulation of bronchopulmonary C-fiber afferents by inhaled cigarette smoke (238). Indeed, TRPA1 is involved in the neurogenic inflammation and tachykinin release in the airways evoked by inhaled cigarette smoke or cigarette smoke aqueous extract, presumably resulting from a sustained stimulation of tachykinin-containing airway afferents by ROS and other irritant gases (e.g., aldehydes, etc.) contained in the smoke (12). A recent study has further revealed that nicotine in the concentrations of 0.1 to 1 mmol/L can activate TRPA1 expressed in mouse trigeminal neurons, but the authors carefully cautioned that this TRPA1 activation mechanism may not be involved in the airway irritation generated by inhaled cigarette smoke because a substantially higher concentration of nicotine was required to activate TRPA1 (352).

Afferents sensitive to other modes of physiological stimuli

In addition to the mechanical and chemical sensitivities, bronchopulmonary afferents process other modes of sensory properties. Among the nonchemical and nonmechanical activators, the most notable ones are temperature and osmolarity, both of which can change substantially under various unusual physiological conditions.

Temperature-sensitive afferents Although the lung is enclosed in the thoracic chamber and constantly exposed to body temperature, an increase in lung temperature can occur under both normal and pathophysiological conditions. For example, body core temperature exceeding 41°C has been reported in healthy humans and animals during exertional exercise (32, 248, 303). Body temperature higher than 40.5°C occurs frequently in patients suffering from severe fever or heatstroke (29), while tissue inflammation can lead to an increase in local temperature in the inflamed area (129, 300). Indeed, a recent report showed that the end-expiratory temperature plateau was 2.7°C higher in mild allergic asthmatic children than in healthy children, and the difference was closely correlated with the exhaled nitric oxide concentration as well as the sputum eosinophil percentage (291). An earlier study also reported a faster rise of exhaled temperature in adult asthmatics than matching controls (287). Taken together, these findings suggest that the tissue temperature increases in the inflamed airways.

All four subtypes of TRPV channels, TRPV1-4, are known as the primary sensors detecting warm and hot temperatures in mammalian species with different temperature thresholds (>43°C for TRPV1; >52°C for TRPV2; >34-38°C for TRPV3; >27-35°C for TRPV4) (82). Recent studies have demonstrated the expression of both mRNA and channel proteins of all these four subtypes of TRPVs in the cell bodies of sensory neurons innervating the lung (268). Although thermal sensitivity is a distinct transduction property of the TRPV1 receptor (50, 372), its importance in regulating the normal function of airway sensory nerve has been largely overlooked, probably due to the fact that the average body temperature is considerably lower than the activation threshold (43°C) previously reported in the heterologously expressed TRPV1 channel (50).

A recent study by Ruan and co-workers reported the first evidence that vagal pulmonary C-fibers were activated and sensitized by increasing the intrathoracic temperature using an isolated thoracic chamber in anesthetized rats (317). However, these effects of hyperthermia on sensory nerves could result from indirect effects via local release of inflammatory mediators (39), cytokines (29, 181), and/or acidosis caused by increase in tissue metabolic rate and CO_2 production because some of these endogenous substances (e.g., prostaglandin E_2, hydrogen ion, etc.) are known to activate C-fiber endings (29, 226). In a subsequent study, Ni et al. have further demonstrated a distinct temperature sensitivity in isolated vagal pulmonary sensory neurons using a whole-cell patch-clamp electrophysiological recording technique (268). When temperature was increased to above 34.4°C, an inward current (in voltage-clamp mode) or membrane depolarization (in current-clamp mode) began to emerge in isolated rat pulmonary sensory neurons. Further increase in temperature increased the amplitude of inward current sharply, or evoked action potential as the temperature reached 39 to 40°C. The temperature coefficient, Q_{10}, was 29.5 over the range of 35 to 41°C and distinctly higher than that over the lower temperature range (23 to 30°C, $Q_{10} = 2.84$), which clearly illustrates the thermal sensitivity of these pulmonary sensory neurons (157). Approximately 48% of the response was blocked by pretreatment with capsazepine, suggesting an important role of the TRPV1; the remaining response probably involves other TRPV channels because it was abolished by ruthenium red, a nonselective TRPV and calcium channel blocker (268).

Furthermore, increasing temperature can also elevate the sensitivity to TRPV1 activators. When temperature was raised from normal (~36°C) to hyperthermic (~40.6°C) level and held constant, the inward current evoked by capsaicin was markedly potentiated in isolated rat vagal pulmonary sensory neurons (269) (Fig. 12). This potentiating effect was clearly present even at a moderate level of hyperthermia (~39°C). Surprisingly, although hyperthermia also potentiated the TRPV1-mediated response to H^+, it inhibited the responses mediated through ASICs (269). The hyperthermia-elevated response to 2-aminoethoxydiphenyl borate (2-APB), a nonselective activator of TRPV1-3 receptors, was largely attenuated by selective TRPV1 antagonists, capsazepine, or AMG 9810 (269), and completely absent in pulmonary nodose and jugular neurons isolated from TRPV1-null mice (270). Clearly, a positive interaction between hyperthermia and chemical activators exists at the TRPV1 channel, but the specific site(s) and underlying mechanisms are not known.

Although the TRPV1 channel is generally considered to be voltage independent, a recent study has clearly shown that physiological stimuli of TRPV1, such as high temperature, can shift its activation curve (open probability vs. voltage) from a nonphysiological positive voltage range towards the negative potential (273) (Fig. 13). This left shift of the activation curve to the physiologically relevant voltage range with a relatively small gating charge can be an important mechanism underlying the functional properties of TRPV1 and its temperature-dependent sensitivity to a diverse range of biological stimuli (273, 372).

Osmolarity-sensitive afferents The osmolarity of airway fluid lining the airway mucosal surface can change under various physiological conditions. For example, inspiration of dry air causes a hyperosmotic shift in the fluid due to water evaporation from the epithelial surface, which is even more pronounced during exercise when air is inhaled at a higher flow rate through the mouth and bypassing the nasal turbinate. Under pathophysiological conditions, the osmolarity of airway surface fluid is significantly decreased in certain airway

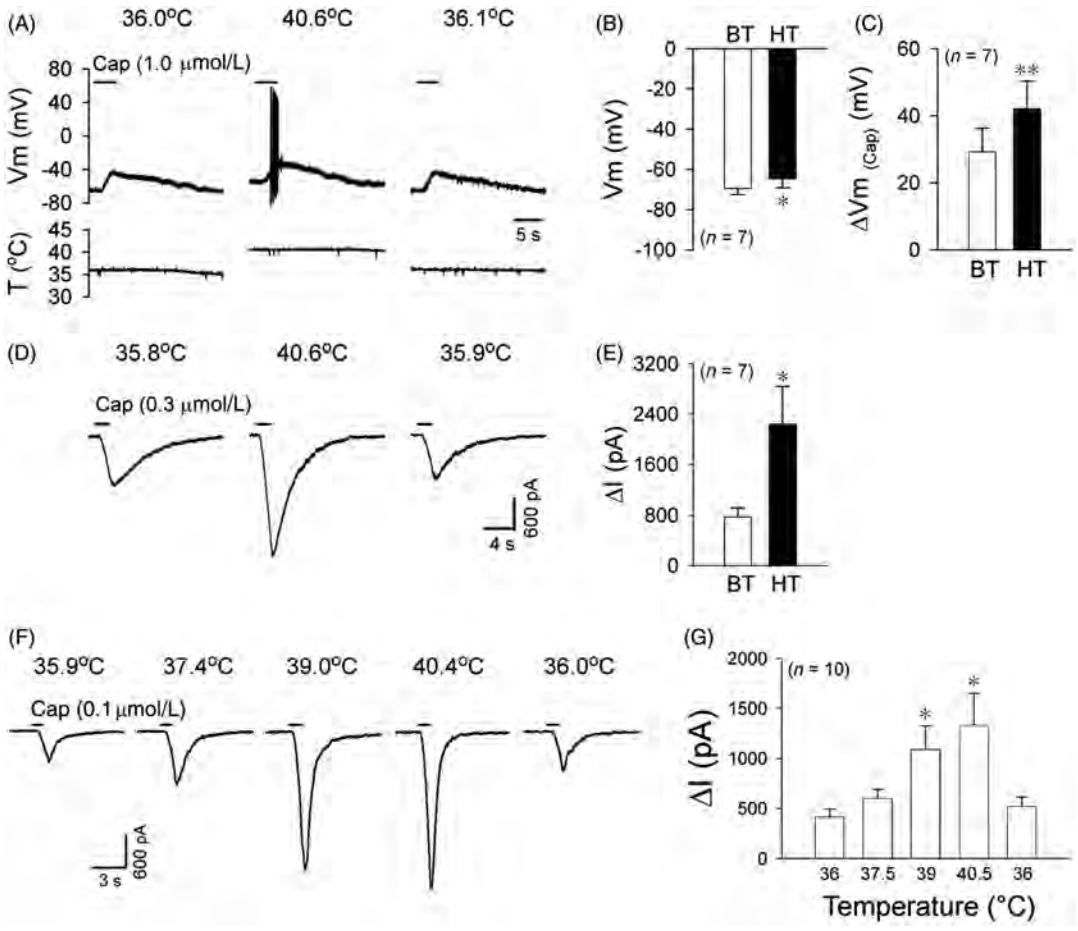

Figure 12 Effect of increasing temperature on the response of vagal pulmonary sensory neurons to capsaicin (Cap). (A) Experimental records illustrating that both membrane depolarization and number of action potentials evoked by Cap (1 μmol/L, 4 s) were increased in current-clamp mode when the temperature was increased from 36.0 to 40.6°C in a jugular neuron; the response recovered when the temperature was returned. V_m, membrane potential; T, temperature. (B) Group data for the baseline membrane potential at the two different temperatures: BT, body temperature (35.7 ± 0.09°C); HT, hyperthermic temperature (40.5 ± 0.11°C). (C) Group data for Cap (0.3-1 μmol/L, 1-8 s)-evoked membrane depolarization, $\Delta V_{m\,(Cap)}$, at the two temperatures. (D) Experimental records illustrating that the Cap (0.3 μmol/L, 2 s)-evoked current was increased when the temperature was increased from 35.8 to 40.6°C in a nodose neuron in voltage-clamp mode. (E) Group data for the Cap (0.3 μmol/L, 2-4 s)-evoked current response (ΔI) at the two different temperatures. (F) Experimental records illustrating that Cap (0.1 μmol/L, 1 s)-evoked current was increased progressively in a jugular neuron when temperature was elevated from 35.9 to 40.4°C by successive steps of ~1.5°C. (G) Group data for the Cap (0.1 or 0.3 μmol/L, 1-3 s)-evoked current response at the five different temperatures as indicated. *$P < 0.05$ and **$P < 0.01$ compared with the corresponding response at BT (36°C). [Modified, with permission, from reference (270).]

diseases due to an increase in glandular secretions and water filtration (e.g., asthma) (185). Conversely, hypertonicity of airway fluid occurs in patients with cystic fibrosis due to the dysfunction of transmembrane conductance regulator protein (131). The osmolarity of body fluid can also increase substantially as a result of systemic dehydration.

An abnormal change in osmolarity in the airway fluid is known to elicit cardiorespiratory reflex responses such as bradycardia, arterial hypotension, apnea followed by rapid shallow breathing and bronchoconstriction; all these effects are abolished by bilateral vagotomy, indicating an involvement of bronchopulmonary afferents (225). Indeed, inhalation of hypertonic saline aerosol has been used as a tussive agent in clinical lab for many years, because of its direct or indirect stimulatory effect on "cough sensors" (242). Inhalation of distilled water aerosol also triggers cough and reflex bronchoconstriction in asthmatic patients (241, 339).

Experimental evidence further demonstrated that both bronchopulmonary C-fiber afferents and RARs were stimulated by instillation of a small amount of hypertonic saline (1200-2400 mmol/L) into the airways, but not by isotonic saline or glucose solution in anesthetized dogs (296, 297). Hypertonic saline also stimulated HTARs in rabbit lung and airways (402). These afferents responded to hyperosmotic saline in a concentration-dependent manner. A similar osmolarity sensitivity was found in C-fibers of the guinea pig trachea and main stem bronchi (108). In addition, application of a small volume of hypoosmotic solution (e.g., distilled water)

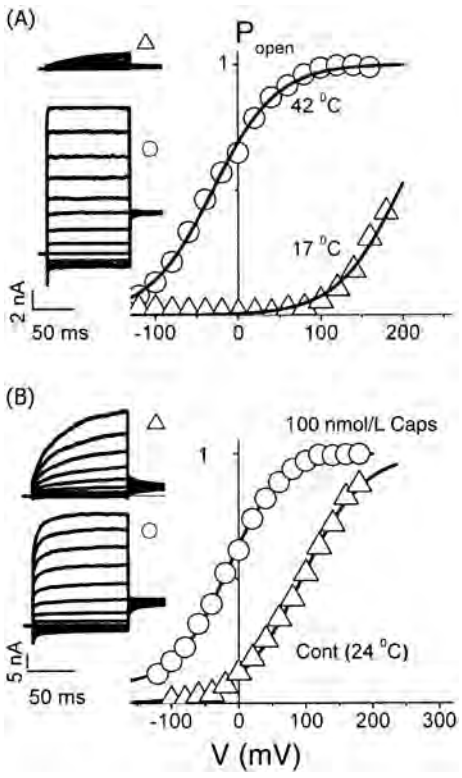

Figure 13 Voltage shifts of TRPV1 activation curves by temperature and capsaicin. (A) Voltage dependence of the open probability of TRPV1 channels at 17°C (triangles) and 42°C (circles). The inset shows the respective current families obtained from a voltage-step protocol (holding potential 0 mV, voltage steps of 100-ms duration from –120 to +160 mV in 40-mV increments). Note the leftward shift of the activation curve toward negative potentials by increasing the temperature. (B) The same voltage protocol as in A, and the temperature was held at 24°C. Activation of TRPV1 by capsaicin (100 nmol/L; circles) also caused a pronounced leftward shift of the activation curve. [Modified, with permission, from reference (273).]

into the airway lumen stimulates RARs (297) and laryngeal afferents (11). The transduction mechanism(s) involved in the osmolarity-sensitive properties of these afferent endings is not fully understood, but one major cause is a lack of permanent anions (e.g., chloride ion) in the fluid (11, 326).

TRPV4, a member of the TRPV channel family, is a tetrameric membrane protein and widely expressed in mammalian tissues including the epithelial cells and smooth muscles in the lung (141). TRPV4 is considered as a sensor for detecting not only warm temperature (27-35°C), but also a change in osmolarity (232, 347). Expressions of both mRNA and protein of TRPV4 have been recently demonstrated in the rat pulmonary sensory neurons (268). Activation of this channel can evoke Ca^{2+} influx and excitation of the neuron. It is well documented that TRPV4 is not only activated by hypotonicity (347), but also by a mild increase in osmolarity, especially at the presence of tissue inflammation (8, 9). Thus, these responses exhibited by heterologously expressed TRPV4 channel may help to explain the stimulatory effects of hypertonic saline and distilled water on airway C-fibers and RARs in the *in vivo* experiments (297).

Polymodal afferents

Majority of bronchopulmomary afferents exhibit polymodal sensitivity. In particular, both bronchial and pulmonary C-fibers exhibit chemical, mechanical and thermal sensitivities (67, 158, 225, 317); the same sensory fibers can be activated by chemical activators (e.g., capsaicin), mechanical means (e.g., light probing or hyperinflation of the lungs), and a change in temperature. Similarly, polymodal sensitivity has also been shown in a subgroup of RARs and laryngeal afferents. These observations are expected since multiple types of receptor proteins have been identified in a single pulmonary sensory neuron, whereas the same receptor protein can exhibit different sensory modality; for example, the cloned TRPV1 expressed in oocytes can be activated by both chemical (capsaicin, proton, etc.) and thermal stimulation (50).

Difference in afferent phenotypes related to ganglion origin

Coleridge and co-workers first described the two separate subgroups of the C-fibers innervating the respiratory tract based upon circulatory accessibility: pulmonary C-fibers (sensory terminals often located in intrapulmonary airways and lung) and bronchial C-fibers (often in the extrapulmonary airways) (see page 5 for detailed descriptions) (67). In addition to the difference in the anatomic structures of their innervation, these two groups of afferents appear to differ in their sensitivity to different types of stimuli. Pulmonary C-fibers seem to be more sensitive to mechanical stimuli (e.g., lung inflation), whereas bronchial C-fibers have greater chemosensitivity (67). However, it is difficult to evaluate the difference quantitatively because the actual level of stimulus at the receptor terminal cannot be accurately measured. Furthermore, vascular anastomosis has been shown to exist between bronchial and pulmonary circulations (52).

A series of recent studies by Undem and co-workers has revealed that this difference in sensory modality between bronchial and pulmonary C-fiber afferents may be related to their different ganglion origins (364). The cell bodies of afferent nerves innervating the respiratory tract are located in two separate but adjacent vagal sensory ganglia: nodose and jugular ganglia. The neurons within these two ganglia have distinct embryological origins: those in the nodose ganglion are derived from the epibranchial placodes, whereas jugular ganglion neurons are derived from the neural crest. Electrophysiological studies have shown that the chemosensitive C-fibers innervating the trachea and major bronchi (bronchial C-fibers) arise almost exclusively from jugular ganglia (314). In contrast, the mechanoreceptors, RARs and SARs, located in intrapulmonary airways and lung parenchyma are derived mainly from nodose ganglion neurons (43, 89). C-fibers innervating deeper regions of the lung arise from both jugular and nodose ganglia (364). Furthermore, these afferents of different ganglion origin also differ in their neurochemical profiles; nearly all the jugular C-fiber neurons innervating the trachea

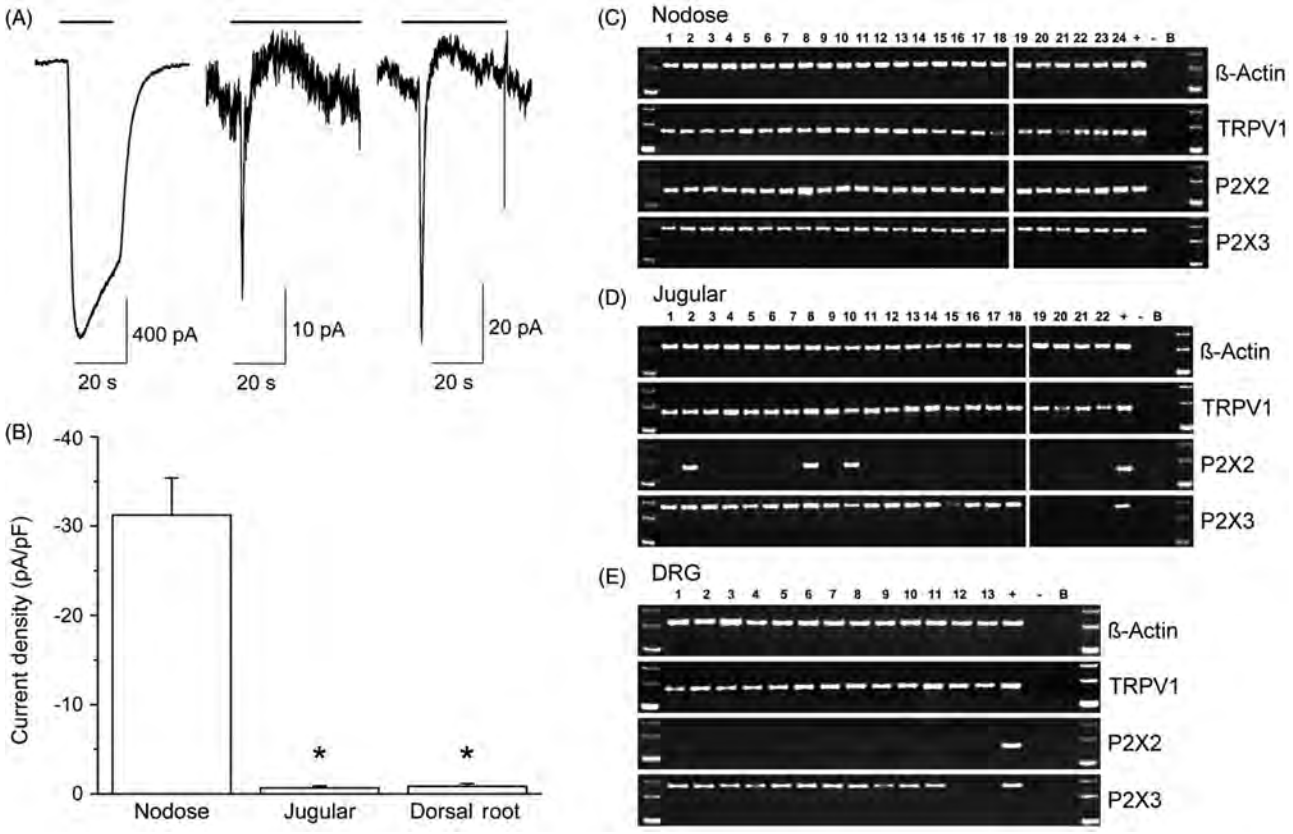

Figure 14 Expression of P2X2 and P2X3 receptors in capsaicin-sensitive pulmonary neurons isolated from nodose ganglion but not from jugular or dorsal root ganglia. (A) Inward currents were elicited by α,β-methylene ATP (30 μmol/L; bars) in pulmonary (DiI-labeled) nodose ganglion (left), jugular ganglion (middle), and dorsal root ganglion (DRG; right) neurons. Neurons were capsaicin sensitive. Standard ruptured patch whole cell recordings were made at room temperature. Holding potential was –60 mV. (B) Comparison of peak α,β-methylene ATP-elicited current density revealed a significant overall difference among the three populations of neurons (1-way ANOVA, $P < 0.001$); and significant differences between nodose ($n = 10$) and jugular ($n = 14$) populations ($P < 0.05$; Tukey test) and between nodose and DRG ($n = 7$) populations ($P < 0.05$). Single-cell RT-PCR analysis of 24 lung-specific nodose neurons (C), 22 lung-specific jugular neurons (D), and 13 lung-specific DRG neurons (E). Message for P2X2 and P2X3 was analyzed in TRPV1-positive neurons. β-actin was used as a positive control. Top, middle, and bottom bands of the ladder represent 300, 200, and 100 bp, respectively. Lanes +, –, and B indicate positive control from whole trigeminal ganglion, no-RT control, and a representative bath control, respectively. [Modified, with permission, from reference (206).]

and bronchi contain SP and CGRP, whereas only a subset of nodose C-fiber neurons contain these neuropeptides (364). In addition, although capsaicin stimulates both nodose and jugular C-fibers, only nodose C-fibers are activated by selective P2X receptor agonist (β-methylene ATP) and express P2X2 receptor mRNA (206, 364) (Fig. 14). A recent study using the single-neuron RT-PCR technique further demonstrated that the TRPV1-expressing jugular C-fiber neurons innervating trachea and lung preferentially expressed the neurotrophin receptor, tropomyosin-receptor kinase A (TrkA) receptor, whereas the nodose neurons expressing TRPV1 innervating intrapulmonary structures preferentially expressed TrkB (233). These distinct differences in the electrophysiological and pharmacological properties, neurochemical and gene expression profiles between the neurons from these two ganglia indicate two different phenotypes of sensory nerves innervating the respiratory tract (364). In mice, there is only one single ganglionic structure where the cell bodies of all first-order vagal sensory neurons are located. Nonetheless, two different phenotypes of pulmonary sensory neurons, located in different region of the ganglion, can be identified based on their afferent properties and gene expression profiles, in a pattern similar to that found in guinea pigs (266).

Reflex Responses

Activation of airway sensors elicits profound cardiopulmonary responses. Identifying the reflex components of a particular type of sensors is complicated because any given stimulus may activate more than one type of sensors, and activate multiple reflex pathways and effectors. Some sensors may be directly activated by the stimulus while others may be secondarily activated by the changes in mechanical or chemical conditions resulting from the primary stimulus. Nevertheless, certain reflex effects can still be delineated. For example, in anesthetized rats, inhalation of airway irritants (375) or ROS (318) elicits an immediate apnea, bradypnea, or

augmented inspiration, and frequently a delayed hyperpnea (e.g., after inhalation of cigarette smoke). Selective blocking the conduction of vagal unmyelinated C-fibers with perineural capsaicin treatment eliminates the immediate inhibitory effect but enhances the augmented inspiration (375). Selective blocking the myelinated afferents by cooling the cervical vagi to 7°C abolishes the augmented breaths and prolongs the immediate inhibitory effect (216). Bilateral vagotomy eliminates both immediate and delayed responses. These results clearly demonstrate that unmyelinated and myelinated afferents mediate the immediate inhibitory and excitatory responses, respectively. At a given time point, respiration is influenced by inhibitory and excitatory reflex effects concomitantly.

Regulation of respiratory system

The activities generated from the pulmonary afferents provide information for the CNS to regulate following functions of the respiratory system.

Breathing pattern

Activation of each type of airway sensors generates specific changes in breathing patterns. Activation of SARs produces Hering-Breuer reflex to suppress inspiration and to initiate expiration. This reflex operates in humans, as the respiratory rhythm entrains to ventilator frequencies during mechanical ventilation (342). However, the reflex is relatively weak in humans because lung inflation above a threshold volume of 50% of inspiratory capacity is required to prolong expiratory time (177). This vagally mediated prolongation is confirmed by its absence in bilateral lung transplant patient. The SAR activity during lung inflation acts as an inspiratory off-switch to cut short inspiration and thus to control inspiratory time and tidal volume (65, 332). Alteration in breathing pattern by activation of SARs is also observed in *in vitro* preparation, in which the isolated neonate rat brain stem was connected with the lungs through the vagus nerves (260). Stimulation of SARs also activates expiratory muscle (247). This vagally mediated activation of expiratory muscle was confirmed in exercising dogs with a reversible vagal blockade (5). The reflex sensitivity is enhanced as respiratory drive increases, which may partially explain the active expiration during exercise and in patients with chronic obstructive lung disease. Activation of RARs triggers augmented breaths (65). Since RARs have little activity during eupnea, they were thought unimportant in normal breathing control. However, low discharge could exert a tonic action on the respiratory center. The RAR activity increases and is synchronized to discharge at the lung inflation phase when the lung compliance is decreased (298). Thus, RARs may signal the need to expand the lungs to avoid atelectasis. Activation of DARs stimulates inspiration and inhibits expiration (403). Activation of HTARs evokes the excitatory lung reflex, which produces tachypnea and hyperpnea, increased inspiratory time and inspiratory duty cycle, shortened expiratory time, and reduced expiratory activity. All these effects stress the inspiratory muscles, resulting inspiratory muscle fatigue (412). Stimulation of C-fibers at low level of intensity produces rapid shallow breathing, but at high intensity causes apnea (65).

Airway smooth muscle

A primary role of vagal bronchopulmonary afferents in the reflex regulation of airway smooth muscle tone has been extensively discussed in recent reviews (40, 366). Activation of SARs can cause bronchodilation and profoundly inhibits laryngeal, tongue, and hyoid muscles (15). Activation of RARs is believed to constrict airways. However, this view is debatable, because this belief is based on that activation of RARs is accompanied by bronchial constriction under several conditions and so far there is no convincing direct evidence to support that activation of RAR can elicit a reflex bronchial constriction (404). Furthermore, some evidence indicates that activation RARs is not accompanied by bronchial constriction (416). Activation of C-fibers causes reflex airway smooth muscle contraction (63) via cholinergic efferents in dogs and cats (65). In anesthetized guinea pigs, intravenous administration of histamine or bradykinin increases tracheal tone, which can be blocked by vagotomy or atropine (45). Increasing temperature at receptive field can activate C-fibers by stimulation of TRPV1 expressed on the airway afferents. An increase in airway temperature by hyperventilation of warm humid air induced an immediate bronchoconstriction and coughs in patients with mild asthma (6, 155) and bronchoconstriction in anesthetized guinea pigs (235). The increase in airway resistance, but not cough, was prevented by ipratropium in asthmatics, suggesting an involvement of cholinergic reflex and an activation of TRPV1-expressing C-fiber afferents innervating the airways (155) (Fig. 15). Whether stimulation of HTARs affects airway tone has yet to be determined.

A distinct difference in the bronchomotor response to pulmonary C-fiber stimulation between different species should be noted (97, 168, 184, 333). For example, intravenous injection of capsaicin evokes dose-dependent bronchoconstriction in dogs, cats, guinea pigs, and hamsters via the centrally mediated cholinergic reflex pathway and/or the local "axon reflex" responses (67, 225, 333, 348); in contrary, capsaicin causes either bronchodilation or no effect in rats and mice, resulting from the tachykinin-induced secondary release of bronchodilating mediators such as prostaglandin E_2 and nitric oxide in the airways (81, 231, 333, 349, 350). A more extensive discussion of this subject is presented in recent reviews (40, 366).

Airway secretion

Tickling the trachea evokes a powerful vagal reflex mediated airway secretion, which may result from multiple sensors. Stimulation of SARs provides no substantial effects on airway secreation, because hyperinflation of the lung does not alter

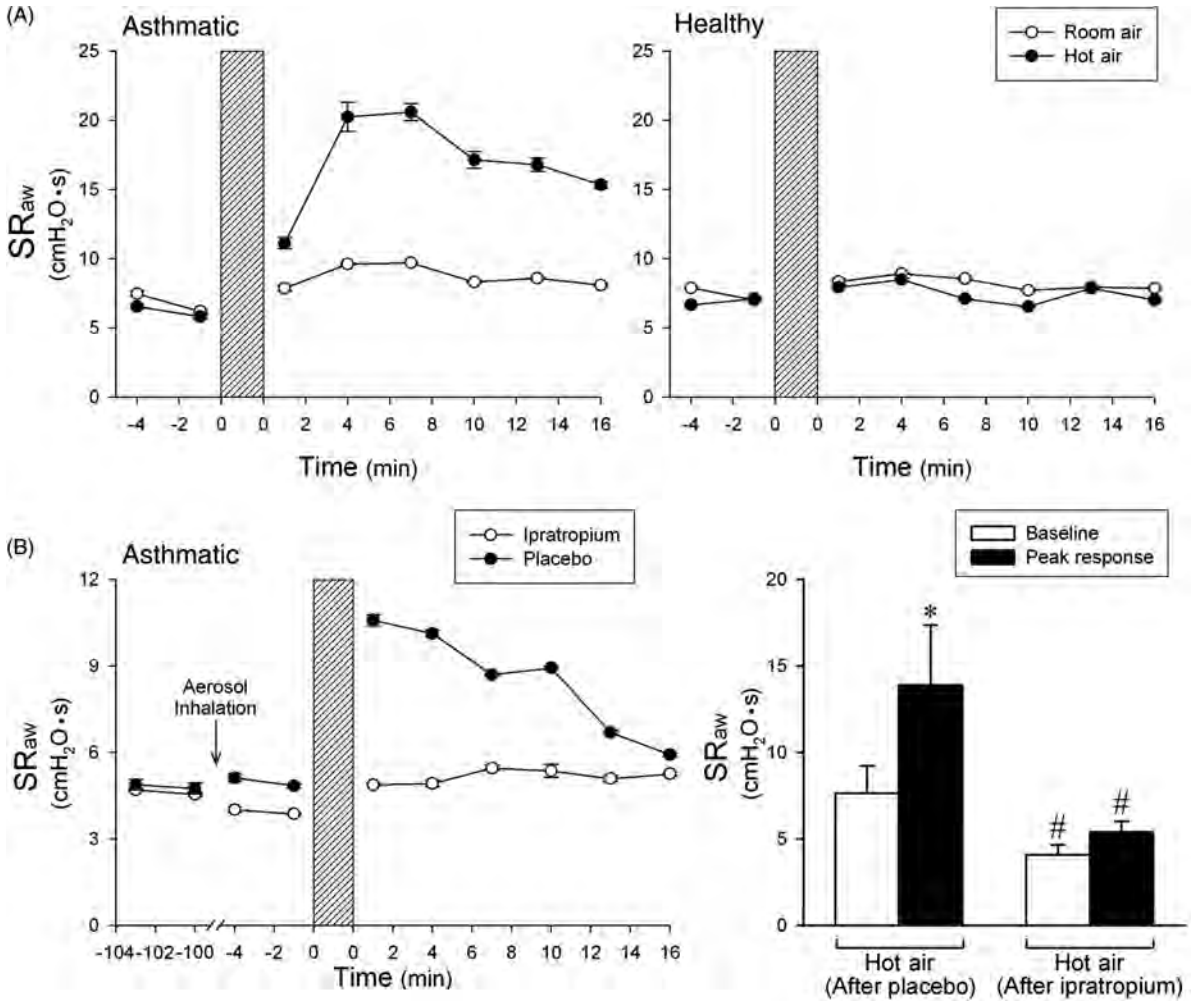

Figure 15 Involvement of cholinergic reflex in the bronchoconstriction evoked by increasing airway temperature in patients with asthma. (A) Representative responses of specific airway resistance (SR_{aw}) to hyperventilation of humidified room air (open circles; 20-22°C, 65-75% relative humidity) and hot air (closed circles; 49°C, 75-80% relative humidity) in an asthmatic patient (left) and a healthy subject (right). Each point represents the data averaged over four consecutive breaths. During hyperventilation (shaded bars), the subjects breathed at 40% of maximal voluntary ventilation for 4 min of a gas mixture of 4.5% CO_2 balance air. Only one experiment was performed in each subject on the same day. (B) Effect of pretreatment with ipratropium aerosol (500 mg; 2.5 ml of 0.02% solution) on hot humid air-induced bronchoconstriction in asthmatic patients. Left: representative SR_{aw} responses obtained in an asthmatic patient. Ipratropium and placebo aerosols were administered in a double-blind fashion, and the response to hot air challenge was tested 90 min after the aerosol inhalation; the two tests were performed about a week apart. Right: average data collected from all six asthmatic patients comparing the peak SR_{aw} responses to hot humid air challenge after ipratropium and placebo pretreatments. Baseline and peak SR_{aw} were averaged over eight and four consecutive breaths before and after hyperventilation of hot air, respectively, in each subject. * denotes significantly different ($P < 0.05$) from the baseline. # denotes significant difference ($P < 0.05$) comparing the corresponding data between ipratropium and placebo pretreatments. Data are means ± SEM. [Modified, with permission, from reference (155).]

the secretion. Activation of RARs may stimulate mucosal secretion because decreased lung compliance, a selective stimulus to RARs, increases the secretion (411). Since lung deflation induced by removal of positive end-expiratory pressure or applying negative pressure increases mucous secretion substantially (411), activation of DARs may stimulate airway secretion. Activation of C-fibers also reflexively stimulates submucosal gland secretion in the trachea (79, 334) and larynx (154). Airway secretion is an important defense mechanism. Inhaled irritant substances may deposit in airway wall, be trapped in the airway secretion, and then expelled by mucocilliary transport and cough. Indeed, activation of the C-fibers also reflexively stimulates the beat frequency of the airway cilia (95), which is attenuated by blocking the reflex pathway with hexamethonium or atropine.

Cough reflex

Coughing is a powerful defense mechanisms and one of the most common symptoms of disease in the lower airway. It is also one of the most complex reflex responses resulting from activation of airway sensors (see page 6). In humans, cough is

often initiated from an irritant sensation in the airway, which is significantly suppressed during sleep (169) and under anesthesia (274). Cough reflex has been frequently reviewed (41, 44). In the rabbit, after selectively blocking SARs by SO_2, cough is no longer present, probably because the effective activation of expiratory muscle is impaired (65). Although activation of SARs does not initiate cough, its action is probably required. Clearly, a full fledged cough action may come from activation of multiple types of airway sensors.

Regulation of cardiovascular system

Circulatory and respiratory systems are coordinated to accomplish an important task to deliver oxygen and remove carbon dioxide. It is not surprising that activation of airway sensors can initiate cardiovascular responses and alter hemodynamics. Up till now, only SARs and C-fibers have been identified to significantly influence cardiovascular system. The roles played by the other types of airway sensors have not been clearly identified. If they do regulate hemodynamics, their effects are probably not as prominent as those initiated from SARs and C-fibers.

Cardiac function

Sensory input from SARs contributes to respiratory sinus arrhythmia (RSA). In healthy subjects, RSA was significant during eupnea and increased two- to three-fold with increasing tidal volume. In double lung transplant patients, RSA decreased by 50% and no longer responded to increasing tidal volume (351). Thus, vagal feedback from the lungs is obligatory for RSA. Increased SAR input increases heart rate (72). Lung inflation at low pressure (relatively selective for SARs) causes reflex tachycardia, whereas inflation at higher pressure causes bradycardia (189). The tachycardia is due to, in part, a reduction in cardiac vagal efferent activities. Marshall and Metcalfe (249) observed bradycardia and transient vasodilatation in hindlimb skeletal muscle accompanying an augmented breath in rats, and attributed them to activation of RARs. However, a high level inflation of the lung can also activate C-fibers, which is known to decrease cardiac output through chronotropic and inotropic inhibitory effects. Bradycardia is mainly mediated through vagal efferents, because the reflex effect is largely blocked by atropine (73), although sympathetic withdrawal may also be a contributing factor (281). In addition, acute lung injury can cause sustained reflex cardiovascular depression, which can be blocked by lung denervation, suggesting an involvement of C-fibers (10).

Vascular resistance

Activation of C-fibers causes significant systemic hypotension, which results from vasodilation in addition to bradycardia and decreased stroke volume (65). The vasodilation occurs in both pulmonary and systemic vascular beds (73); the latter includes those in coronary (281), renal (369), tracheal (322, 382), bronchial (295), and hindlimb (49) circulations. Withdrawal of sympathetic efferent activity is responsible for renal vasodilation (369). An active, cholinergic component may also play a role in the reflex vasodilation. For example, in rats, hypotension evoked by right atrial injection of phenylbiguanide was abolished by blocking both cholinergic and α-1 adrenergic receptors (337), and the coronary vasodilation is largely eliminated by atropine (281). It is long known that activation of SARs can cause systemic vasodilation. Daly et al. examined the effects of changing tidal volume on systemic vascular resistance in open chest mechanically ventilated dogs using extracorporeal circulation to keep blood gases at constant levels. They found that increase in tidal volume decreases the resistance (74). The decreases in vascular resistance induced by lung inflation are mainly mediated by withdrawal of sympathetic activity (410). Such a reflex exists in human. For example, lung inflation-induced alteration in sympathetic activity to the muscle is abolished in lung transplant patients (336).

Bronchial circulation

Bronchial circulation deserves a special consideration because it delivers blood supply to all the airway sensors located in the large airways. Stimulation of C-fibers produces bronchial vasodilation. The increased blood flow can lead to an increase of water content in the airway luminal surface and dilute the irritants that initiate the reflex (295). Pisarri et al. proposed that increased bronchial blood flow is a part of the pulmonary chemoreflex, because it can be evoked along with other chemoreflex responses (apnea, bradycardia, and systemic hypotension) by right atrial injection of capsaicin. Bronchial blood flow was increased by several folds following right atrial injection of capsaicin despite a decrease in its perfusion pressure in anesthetized and mechanically ventilated dogs (61). The capsaicin-induced bronchovasodilation was abolished by vagotomy in approximately half of the dogs studied (295), supporting a major role of the centrally mediated reflex. Assessed from atropine blockade studies, approximately one-half of bronchial vasodilation is mediated through cholinergic vagal efferents in sheep (61) and dogs (295). In sheep, vasoactive intestinal peptide or nitric oxide pathways may contribute to a small extent to noncholinergic parasympathetic bronchial vasodilation and the axon reflexes, whereas the α-adrenergic pathway contributes little to vasodilation (243). Stimulation of SARs does not alter tracheal vascular resistance in dogs (322).

Regulation of other organs/systems

Renal function

Kappagoda and co-workers first demonstrated that obstruction of lymphatic drainage from the lung increases urine flow and sodium excretion in anaesthetized rabbits (311). The diuresis was mediated by vagal pulmonary afferents because the response persisted after both vagi were sectioned

at the level of the diaphragm (259), but was abolished by bilateral cervical vagotomy (311). Furthermore, the increase in urine flow was attenuated after cooling both cervical vagi to 8°C, indicating an involvement of the myelinated lung afferents (311). These investigators believe that RARs were responsible because in their earlier observations that RARs were consistently activated when a mild increase in interstitial fluid volume or pressure in the lung was experimentally increased by pulmonary lymphatic obstruction, mitral valve lesion, or pulmonary venous congestion (142, 150). They further demonstrated that the efferent pathway of reflex diuresis lies in the sympathetic nerves to the kidney, and activation of α_2 adrenergic receptors and release of nitric oxide (420) through the action of neuronal nitric oxide synthase within the renal medulla is likely involved (258). Thus, these myelinated lung afferents by monitoring the extravascular fluid volume in the lung, may play an important role in regulating the body fluid volume and preventing pulmonary congestion and edema (187).

Somatic responses

Paintal reported the first evidence that stimulation of J receptors (pulmonary C-fibers) by intravenous injection of phenyldiguanide reflexively inhibits spinal motor neurons in anesthetized cats, and the response was abolished by a lesion in the caudate nucleus. Paintal termed this response as "J reflex" (282, 283). Pulmonary C-fiber activity can also be elevated by increasing left atrial pressure during pulmonary congestion in anesthetized dogs (59). Pressures in pulmonary arteries and capillaries also increase during strenuous exercise, or as a result of pulmonary vasoconstriction in the hypoxic environment at high altitude. Based on these observations, Paintal hypothesized that stimulation of pulmonary C-fibers inhibits spinal motor neurons and somatic muscle activity (83, 124, 192, 283, 292), which plays an important protective role during exertional exercise or at high altitude by limiting muscular performance, and thereby preventing overexertion and possible development of pulmonary edema (283). To test this hypothesis, Pickar and co-workers produced fictive exercise (walking on a treadmill) in decerebrated unanesthetized cats by electrical stimulation of the mesencephalic locomotor region, and stimulated J receptors by either intravenous injection of phenylbiguanide or inflation of a balloon placed in the left atrium. Stimulation of J receptors abolished or attenuated locomotion, and bilateral cervical vagotomy prevented "the J reflex," suggesting that locomotion can be prevented by a viscerosomatic reflex arising from the lungs. However, Gandevia et al. reported that intravenous bolus injections of suprathreshold doses of lobeline evoked airway irritation in a dose-dependent fashion, but "during sustained voluntary contractions of the elbow flexors at submaximal or maximal levels did not impair the ability to produce force" in healthy human volunteers, which raised the question about the degree of effectiveness of J reflex in inhibiting exercise in humans (118, 119). Whether the voluntary command may have overridden the subcortical inhibitory reflex action in conscious subjects remains to be determined.

Interaction with Immune System

Cross talk between airway sensory nerves and inflammatory cells

Both immune and inflammatory responses are important mechanisms for body defense and therefore must be tightly regulated. An insufficient response provides too little protection while an overreaction induces tissue damage. There is increasing evidence that the brain possesses control of the immune system (127), where neural and immune interactions form a circuitry to reflexively monitor and adjust inflammatory responses. Airway sensory nerves may play an important role in the neural-immune interaction. For example, airway receptors can be sensitized during viral infection (see page 28), and thus, releasing neuropeptides and inflammatory cytokines that enhance airway inflammation and hypersensitivity or lead to injury.

Cross talk between airway nerves and immune cells has been recognized for long time. Nerves in the airways closely contact mast cells (75) and other inflammatory cells (178), and can modulate their function. Stimulation of the vagus nerve enhances antigen-induced mast cell degranulation in canine airways (227), while stimulation of sympathetic nerves suppresses it (120). Tachykinin can regulate mast cell function (338). In the airway wall, eosinophils influx increased following capsaicin and SP infusion. This influx was significantly reduced by pretreatment with neurokinin type 1 (NK_1) and NK_2 receptor antagonists (359). Sensory nerves have a close relation with dendritic cells, providing anatomical basis for neuro-immune interaction (370). In mice, some T cells formed clusters with nerve-contacting dendritic cells beneath the smooth muscle layer. They proliferated only during allergic inflammation but not during control, suggesting that sensory nerves influence dendritic cell-driven T cell activation in the airway (371).

Both innate and acquired immune systems can respond to airway assaults (e.g., allergens and irritant chemicals, etc.), and activate inflammatory cells (e.g., eosinophils, neutrophils, etc.) and release ROS (396). Some of these ROS such as hydrogen peroxide and hydroxyl radicals are known to exert stimulatory effects on pulmonary afferents (319, 320). For example, hydroxyl radicals are responsible for the apneic response elicited by inhalation of gas phase cigarette smoke in anesthetized rats (214). Ruan and co-workers have recently reported that activation of the TRPV1 and P2X receptors by endogenously released COX metabolites and ATP, respectively, is partially responsible for ROS-evoked stimulation of pulmonary C-fiber afferents in rats (319, 320). Several recent studies have further demonstrated that ROS and lipid products of peroxidation can activate TRPA1 channels expressed in a subset of vagal bronchopulmonary C-fibers (25, 353, 355).

Neurons can be stimulated by inflammatory cytokines. Immune cells express SP and its receptors (209), providing

substrates for interaction. CNS neurons express both tumor necrosis factor-α (TNFα) and interleukin-1 (IL-1) (31). Peripherally derived cytokines may stimulate the neuroendocrine system centrally by crossing the blood brain barrier through circumventricular organs, or in the periphery by stimulating vagal afferents and also possibly spinal afferents. The CNS exerts a proinflammatory effect to control the immune system by releasing acetylcholine (ACh), which acts at M_3 receptors on alveolar macrophages, and increase chemotactic activity in neutrophils, monocytes, and eosinophils (331). Conversely, the brain may release ACh for an anti-inflammatory effect on peripheral macrophages to decrease their TNFα production and secretion (360).

Signaling and regulatory molecules

Airway sensory nerves can be stimulated or modulated by a variety of agents, such as chemicals, mediators, peptides, and ion channel modulators (363). Mast cells are the major source for 5-HT, histamine, arachidonic acid metabolites, tryptase (335), and nerve growth factor (NGF) (228). Eosinophils, neutrophils and other inflammatory cells infiltrate into the airways during anaphylactic reaction and mucosal inflammation. A number of low molecular weight, highly cationic, cysteinine/arginine-rich proteins are synthesized and secreted by these inflammatory cells (69, 102, 125) which are known to play a key role in the mucosal injury (112, 149, 401) and pathogenesis of asthma (102, 126, 149). Indeed, intratracheal instillation of eosinophil cationic protein or major basic protein can stimulate vagal pulmonary C-fiber endings and elicit pulmonary chemoreflex responses in anesthetized rats (135, 221). Recent studies have further demonstrated that these eosinophil-derived cationic proteins enhanced the excitability of isolated rat vagal pulmonary chemosensitive neurons to capsaicin, acid and ATP (138,139). In addition, these cationic proteins altered the decay time and recovery phase of the action potentials evoked by current injections, and significantly inhibited both the sustained delayed-rectifier voltage-gated K^+ current (IK_{dr}) and the A-type, fast-inactivating K^+ current (IK_a) in these sensory neurons. These actions generated by the eosinophil-derived cationic proteins may play an important part in their sensitizing effects on the C-fiber sensory nerves (139).

During lung inflammation, in addition to inflammatory cells (mast cells and leukocytes), a variety of neural, epithelial, endothelial, and phagocytic cells can produce chemical mediators that activate airway sensors by sensitization or direct stimulation. These substances include neuropeptides and inflammatory mediators such as tachykinins, CGRP, prostaglandins, bradykinin, adenosine, 5-HT, histamine, and ROS (60, 225, 226). In addition, pro-inflammatory cytokines (interferons, TNFα, IL-1β, IL-6, and IL-8) and anti-inflammatory cytokines (IL-10) are mobilized. Indeed, some of these cytokines are known to play an important part in the pathogenesis of chronic airway inflammatory diseases such as asthma. Local injection of IL-1β into the lung parenchyma near the receptor terminal has also been shown to stimulate both pulmonary C-fibers and HTARs in anesthetized rabbits (407). A recent study has further demonstrated that a pronounced potentiating effect on the responses of isolated pulmonary sensory neurons to TRPV1 activators was induced after incubation with TNFα for ~24 h or longer (171). The relative long duration required for the potentiating effect of TNFα to take place seems to suggest the probability of a transcriptional upregulation of the expression of TRP channels in these neurons. Furthermore, the immunoreactivity to both TNF receptor types 1 and 2 were detected in rat vagal pulmonary sensory neurons (171). The stimulation and increased excitability of these sensory nerves can then cause airway smooth muscle contraction, mucus secretion, increased vascular permeability and inflammatory cell chemotaxis through both centrally mediated reflexes and local axon reflexes (40, 80, 225, 226).

Clinical Relevance

Acute airway inflammation and lung injury

During airway mucosal inflammation, the tachykinin content in nociceptor afferents increases (100). The tachykinins include SP, neurokinin A, and neurokinin B, preferentially stimulating NK_1, NK_2 and NK_3 receptors, respectively. Antidromic electrical stimulation of vagal afferents can cause so-called neurogenic inflammation by releasing SP (80, 244). SP causes airway epithelial cells to release prostanoids, which amplify the initial stimulatory effect by increasing tachykinin release, resulting in the inflammatory reaction. Arachidonic acid is liberated from membrane-bound phospholipids by phospholipase A_2, forming a variety of inflammatory mediators. Under the action of COX, arachidonic acid forms prostanoids [including prostaglandins E and F, prostacyclin (PGI_2), and thromboxanes (such as TXA_2)], whereas the lipoxygenase pathway produces leukotrienes. These mediators can activate or sensitize the sensory receptor in response to chemical or mechanical stimuli (225). COX has constitutive (COX-1) and inducible (COX-2) isoforms. COX-2 can be induced by cytokines and growth factors and is the main source for the production of inflammatory mediators during pulmonary diseases (263). Steroids inhibit COX-2 induction.

SP released by C-fibers may stimulate macrophage to release TNFα (360). Activation of NK_1 receptors upregulates proinflammatory cytokine expression (84). These mediators and cytokines directly stimulate peripheral sensory nerves to initiate or reflexively modulate immune responses, and cause behavioral changes such as malaise and fever (316). Indeed, IL-1β stimulates hepatic vagal afferents after being injected into the portal vein (271), and IL-1 receptors are expressed in neurons in the nodose ganglia (94). Similarly, IL-1β also stimulates abdominal visceral C-fibers in sympathetic afferents, which can be blocked by pretreatment with an IL-1 receptor antagonist (113). Local injection of IL-1 into the receptive fields of pulmonary C-fibers and HTARs

stimulates their afferent activity within a few seconds. This provides direct evidence to support a role for the vagal nociceptor in signaling immune activation to the brain. The vagus nerve may promote an immediate reaction in the host defense response to inflammation and injury by reflexively releasing ACh and proinflammatory mediators. This leads to further stimulation of nociceptors (axon reflexes) and release proinflammatory cytokines and chemokines by local inflammatory cells. Chemokines then attract phagocytes, such as macrophages and neutrophils to engulf pathogens and destroy the invading organisms.

Chronic Airway inflammation

Airway inflammation is a common pathophysiological feature of chronic airway diseases including asthma, chronic bronchitis, and chronic obstructive pulmonary disease (COPD). Increasing evidence suggests that a heightened sensitivity of airway sensory nerves plays an important role in the manifestation of many of the symptoms associated with these airway diseases, such as airway irritation, cough and bronchoconstriction.

Recent studies have revealed some important inflammatory signaling pathways and mediators possibly involved in the airway hypersensitivity. One of the most frequently used experimental animal models of chronic airway inflammation is airway sensitization with allergen. For example, rodents (guinea pigs, mice, and rats) can be actively sensitized by an initial intraperitoneal injection and followed by chronic inhalation of aerosolized ovalbumin, which induces chronic airway inflammation (infiltration of eosinophils and neutrophils), expression of TH2 cytokines and non-specific bronchial hyperreactivity, in a pattern similar to those observed in patients suffering from allergic asthma. Several recent reports showed that the baseline activity and sensitivities of pulmonary C-fibers to both chemical stimulants and lung inflation are markedly elevated in animals sensitized with ovalbumin (205, 418). More interestingly, after chronic inflammation was induced in ovalbumin-sensitized rats, capsaicin sensitivity was detected in some of the vagal myelinated afferents, including both RARs and SARs, which normally do not respond to capsaicin (418). In the meantime, the expression of TRPV1 in bronchopulmonary neurons in nodose ganglia also increased significantly, mainly in neurofilament-positive (myelinated) neurons (418) (Fig. 16). In addition, expression of the TRPV1 receptor is upregulated in the sensory nerves innervating the laryngeal mucosa both in humans and experimental animals exposed to gastroesophageal and laryngopharnygeal reflux (147, 148). The overexpression of TRPV1 may be a contributing factor to the various symptoms (e.g., laryngeal hypersensitivity, cough) in the patients with these reflux diseases (20, 99, 302).

The mechanism(s) possibly involved in this phenotypic change is not known. However, neurotrophins such as NGF and brain-derived neurotrophic factor (BDNF) should be considered because their synthesis and release are known to increase in allergic airways (28, 239). NGF has been shown to increase capsaicin sensitivity of dorsal root ganglion (DRG) sensory neurons (116, 341, 392, 393), probably by increasing the expression of the TRPV1 channel protein (391), promoting translocation of the TRPV1 to cell membrane (57), and modulating the TRPV1 function by releasing the receptor inhibition from phosphotidylinasitol-4,5-bisphosphate (57). In addition, neurotrophins such as NGF, BDNF, neurotrophin-3 (NT-3), and NT-4/5 may play important roles in neuron growth, maturation and function. These neurotrophins have a high affinity to TrkA (NGF), TrkB (BDNF and NT-4/5), and TrkC (NT-3). Each neurotrophin also binds to a low-affinity receptor, p75NTR (172). Overexpression of NGF exhibits an airway hyperinnervation by tachykinin-containing sensory nerve fibers (167), while suppression of low affinity receptor, p75NTR decreases airway innervation (213). In patients with asthma or other types of airway inflammatory reactions, there is a pronounced increase in the NGF level in the serum and bronchoalveolar lavage fluid (28, 279). NGF mediates inflammation-induced airway hyperresponsiveness (323) and may even cause phenotypic switch of airway sensors, making mechanosensors to express the mediators that are typical in the chemosensitive neurons (176). NGF increases expression of ATP-sensitive P2X3 receptors for nociception (309), and may also lower C-fiber threshold to mechanical stimulation (87, 145), making these chemosensors mechanosensitive. Furthermore, production of NGF is stimulated by TNFα and IL-1β but is inhibited by steroids (110), suggesting that NGF may play an important role in airway remodeling (including sensory neurons), especially during chronic inflammatory diseases, such as asthma and COPD. Currently, investigations are focused on the mechanisms of proinflammatory cytokines, including therapeutic strategies targeting at antagonizing these proinflammatory processes. Not enough attention has been paid to upregulating anti-inflammatory cytokines as a strategy to quench the inflammatory process. In reality, the response to an insult is determined by both pro- and anti-inflammatory processes.

The involvement of TRPV1 in the neural plasticity developed during the chronic airway inflammation appears to gain increasing interest in recent studies (220). Cough sensitivity to capsaicin or citric acid aerosol was markedly elevated in patients with asthma or airway inflammation (88, 276). Endogenous TRPV1 activators such as H^+ and lipoxygenase metabolites are consistently detected in the bronchoalveolar lavage fluid, sputum, and/or exhaled breath condensate of patients with airway inflammatory diseases (121, 180). In addition, certain endogenous inflammatory mediators (e.g., prostaglandin E_2, bradykinin, etc.) can markedly enhance the sensitivity of TRPV1 and lower its threshold for activation (220). Recent studies further revealed an increase in expression of the TRPV1 channel in bronchopulmonary sensory nerves in patients with certain chronic airway diseases (133, 262). These observations collectively suggest that plasticity of TRPV1 develops upon the action of various inflammatory

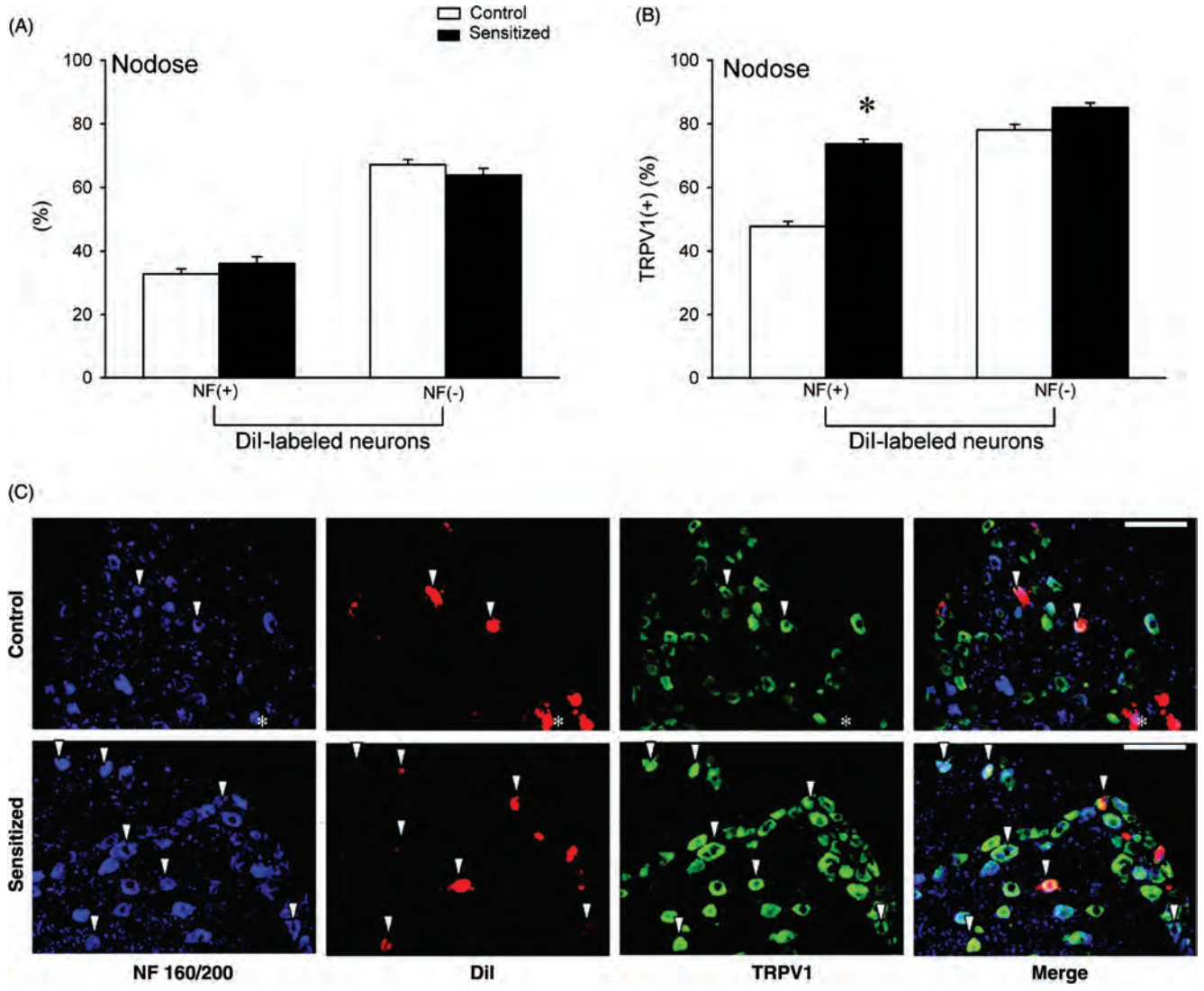

Figure 16 Effect of ovalbumin sensitization on TRPV1 expression in neurofilament (NF)-positive and NF-negative neurons with DiI labeling in nodose ganglion. (A) Percentages of NF-positive and NF-negative neurons in DiI-labeled pulmonary neurons. (B) Percentages of TRPV1-positive neurons in NF-positive and NF-negative neurons labeled with DiI. Open bars, average data obtained from control rats ($n = 4$); filled bars, sensitized rats ($n = 4$). Values are means ± SEM. * denotes $P < 0.05$, significant difference in responses between control and sensitized rats. (C) Representative 20× photographs of triple-labeling immunohistochemistry of NF, DiI, and TRPV1 in nodose ganglia of both control and sensitized rats. The first column from left, NF staining in blue; second, DiI labeling in red; third, TRPV1 staining in green; fourth, the merged image. Arrows are added to depict colocalization of NF staining, DiI labeling and TRPV1 staining in the same neurons. Asterisk depicts the neuron with colocalization of NF staining and DiI labeling, but without TRPV1 staining. Scale bar, 100 μm. [Modified, with permission, from reference (418).]

mediators and cytokines during airway inflammation. In the presence of either an enhanced sensitivity or an increase in expression of TRPV1, a given stimulation will evoke a more intense discharge in these sensory nerves, and consequently heightened reflex responses, resulting in airway hypersensitivity.

Chronic allergic inflammation is also known to increase the expression of preprotachykinin mRNA in the vagal sensory neurons (100), and the amount of tachykinins and CGRP synthesis and release in the airways. Chronic airway inflammation induced by ovalbumin sensitization can also cause a phenotypic switch in the tachykinergic innervation of the airways (264). Under normal conditions, tachykinins are only found in small-diameter, neurofilament-negative neurons innervating the guinea pig airways. However, in ovalbumin-sensitized guinea pigs, SP and CGRP are found in ~30% of the large-diameter, neurofilament-positive neurons. Considering the potent and diverse effects of these sensory neuropeptides on various target cells in the airways, such a phenotypic switch and an increase in the expression of these peptides are likely to play a part in the overall regulation of airway function in allergic asthma (365).

Airway inflammation can be also induced by chronic exposure of airways to environmental air pollutants and irritants. For example, acute inhalation of ozone [0.7-3.0 parts per million (ppm), 1-2 h], an environmental air pollutant, induces

airway hyperresponsiveness to inhaled histamine or acetylcholine in various species including humans (161, 162, 217). The augmented bronchoconstriction is mediated through both cholinergic and tachykinergic pathways, suggesting an involvement of bronchopulmonary C-fibers. Indeed, ozone-induced airway hyperresponsiveness was consistently accompanied by symptoms of coughing and dyspneic sensation in human subjects (161). Coleridge and coworkers first demonstrated that brief exposure to ozone (2-3 ppm) stimulated bronchial C-fibers in dogs (66). Ho et al. further showed that the responses of pulmonary C-fibers to lung inflation and chemical stimulation (capsaicin and lactic acid) were markedly enhanced for a sustained period (45-80 min) after their baseline activity returned to control following exposure to ozone (3.0 ppm; 30 min) in rats (159). A similar pattern of sensitization was also observed when acute airway mucosal damage was generated in other experimental conditions; for example, airway hyperreactivity accompanied by mucosal injury was induced in guinea pigs exposured to toluene diisocyanate (TDI; 1-2 ppm, 1-2 h) (128, 340), an industrial air pollutant known to induce asthma in man after prolonged exposure (288). The TDI-induced hyperresponsiveness was attenuated by treatment with tachykinin antagonists or by depletion of tachykinins in the airways, suggesting the involvement of bronchopulmonary C-fiber afferents (340, 358, 388).

Cigarette smoking is known to cause chronic airway inflammation, accompanied by airway hyperresponsiveness (199). Experimental evidence indicates that enhanced excitability of vagal bronchopulmonary sensory nerves and increased tachykinin synthesis in these nerves resulting from chronic inflammation are important contributing factors to the smoking-induced airway hyperresponsiveness (199). Cigarette smoke causes ROS production, either directly or via the formation of lipid peroxidation products, which activates redox-sensitive signaling pathways and transcription factors resulting in increases in the synthesis and release of inflammatory mediators from various types of structural and inflammatory cells in the airways (55, 308, 362). Some of these inflammatory mediators such as prostanoids are known to exert potent sensitizing and stimulatory effects on pulmonary C-fiber afferents (226).

Airway viral infection

Respiratory viral infections are often accompanied by inflammation and injury of the airway mucosa that is densely supplied by tachykinins-containing, TRPV1-expressing sensory nerves (195, 381). Viral infection is also frequently associated with serious complications such as chronic cough and exacerbation of asthma, especially in young children (123, 293). Neurogenic inflammation in the airways evoked by C-fiber activation with capsaicin was amplified in rats inoculated with respiratory syncytial virus (RSV), resulting from the upregulation of NK_1 receptors in the airway epithelium and vascular endothelium (294). Stimulation of C-fiber sensory nerves causes an overexpression of NK_1 receptors in CD4+ T cells and the chemotactic effect on these lymphocytes during the RSV infection in the airways (14). Furthermore, inoculation of guinea pigs or rats with RSV or Sendai virus (parainfluenza virus) generates long-lasting airway inflammation, promotes overexpression of NGF, and causes a phenotypic switch in tachykininergic innervation of the airways (46, 170, 175). Whether these increases in the airway tachykinin synthesis and expression of NK_1 receptor are accompanied by upregulation of TRPV1 expression in the viral infection-induced airway hyperreactivity (36) is not known. More importantly, whether respiratory viral infection alters the excitability of TRPV1 in the airway sensory nerves also remains to be determined.

Sympathetic Afferents

Sensory information generated by mechanical and chemical stimuli to the airways and lungs is also conducted by sympathetic afferents to the CNS. Technically, it is much more challenging to record sympathetic afferents than vagal afferents, partly due to the accessibility. Therefore, we know much more about the vagal than the sympathetic afferents. It is generally believed that the sympathetic afferents are less important than their vagal counterparts under normal and pathological conditions since most of the known airway reflexes can be significantly suppressed by bilateral vagotomy. However, if these two afferent systems interact synergistically, removing one afferent system may greatly reduce the response generated by the other. Therefore, it would be interesting to know the strength of vagal reflexes following removal of sympathetic afferents.

Anatomic description

Sympathetic afferents travel with their efferent counterpart. Their cell bodies reside in the DRG and fibers travel through the white ramus communicans to the paravertebral ganglia as well as the prevertebral ganglia. The fibers traveled in the middle cervical ganglia, the stellate ganglia and the upper thoracic ganglia supply the lungs and bronchi. After microinjection of Fast Blue tracing dye into the right main stem bronchi of the mouse, Dinh et al. observed that the tracing is mostly terminated at thoracic segments T1 to T6, but also up to C7 and down to T8 (86). Twelve percent of the Fast Blue labeled DRG neurons is immunoreactive to TRPV1. Significant portion of these TRPV1 containing airway neurons also coexpress SP. Interestingly, more than 60% of vagal (nodose and jugular) sensory neurons are also TRPV1-IR (418). Thus, both vagal and sympathetic afferents may contain TRPV1 and SP. Such similarities suggest the possibility of an overlap in their function. After retrograde labeling from the lung, Plato et al. compared the neuron sizes and chemical expressions (combination expression of CGRP and lectin IB4) in the DRG and vagal ganglia. IB4 labels a subpopulation of small, nonpeptidergic nociceptors. They found that no distinction can be made between the two populations of airway neurons in the spinal and vagal ganglia (301). Morphological studies

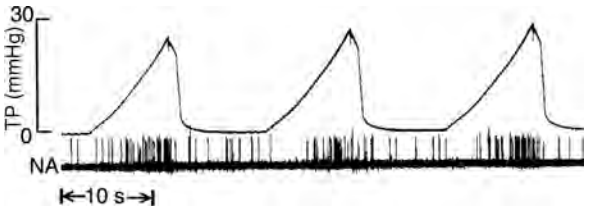

Figure 17 Sympathetic afferent activity recorded from a multifiber preparation in the right second thoracic white ramus. The afferents respond to increases in tracheal pressure during positive-pressure ventilation. Signal-to-noise ratio was electronically enhanced. TP, tracheal pressure; NA, nerve activity. [Modified, with permission, from reference (198).]

demonstrate the presence of sympathetic afferents within the lungs (204, 245, 307). However, up till now, there is no clear description regarding morphological structures of the airway sympathetic sensors.

Receptor subtypes and afferent properties

Sympathetic sensory activity has been recorded in stellate ganglia (160) and white rami of T2 to T4 (198). This activity increases during lung inflation (Fig. 17). Thus, some sympathetic sensors must be mechanosensitive. It is possible that these sensors are corresponding to the receptor structures found in the visceral pleura. As lung expands, elastic fibers in the pleura are stretched, and the sensors are activated. The neurons in the deeper laminae of the thoracic spinal cord are believed to process nociceptive information from the lower airways. Ammonia and smoke produced excitatory, inhibitory, and biphasic responses in these neurons (173). Recently, Qin et al. characterized the pulmonary sympathetic sensors. Their results showed that inhaled ammonia and/or hyperinflation of lungs not only activate deeper neurons but also excite superficial neurons in the spinal cord at T3 level. Bilateral cervical vagotomy does not affect the superficial neurons' responses, but alters the responses of deeper neurons (304). These airway neurons receive the modulatory inputs from distant spinal segments, because their responses to ammonia are suppressed by electrical and chemical stimulation of C1 and C2 spinal neurons. Comparing with the vagal afferent system, sympathetic afferents have much more diffused sensory territory. For example, sensory fields arising from different organs may activate a single thoracic sensory unit (18). Inhaled ammonia not only activates thoracic neurons but also modulates activity of distant colorectal neurons (lumbosacral spinal cord) (306). Majority of the T3 ammonia-sensitive neurons are also activated by noxious stimuli from somatic fields in the chest and upper back areas, as well as from other visceral organs such as the heart and the esophagus (305).

So far there is no report dividing sympathetic sensors into different subcategories. However, based on the afferent properties and reflex studies more than one sympathetic afferent pathway exists (see next paragraph). Sympathetic sensors are known to be stimulated by bioactive agents, such as bradykinin, ammonia, cigarette smoke (173). Effects of bradykinin are mediated by B_2 receptors (345). Evidenced by electrical recording, some T3 spinal sensory neurons respond to ammonia inhalation. The response can be abolished by resiniferatoxin to debilitate TRPV1 containing sensors (304). Taken together, the sympathetic sensors in the lung are a heterogeneous group and are polymodal nociceptive sensors that participate in various pathological processes.

Reflex responses

Stimulation of pulmonary sympathetic afferents alters breathing pattern. For example, after bilateral vagotomy, bradykinin injected into the bronchial artery evokes irregular spasmodic inspirations abolished by avulsion of the upper thoracic sympathetic chain at T1 to T5 (62). Right atrial injection of bradykinin stimulates breathing in vagotomized rabbits (151) and rats (224). However, electrical stimulation of pulmonary sympathetic afferents is reported as having no effect on diaphragm activity (68) or inhibiting it (197). In vagotomized rabbits, activation of pulmonary sympathetic afferents by directly injecting bradykinin into the lung parenchyma produces profound cardiopulmonary reflex effects, including stimulated inspiration and expiration, bradycardia, and hypotension (346, 376). Furthermore, bradykinin elicits both respiratory excitation and inhibition (346), supporting multiple pathways in the sympathetic afferent system. Recently, a sympathetic reflex pathway in guinea pig airways has been described. The afferents arising from the thoracic DRG are capsaicin-sensitive and contain SP. The efferent nerves arising from both thoracic and superior sympathetic ganglia cause relaxation of airway smooth muscles via activation of β-adrenoceptors (278).

Clinical relevance

Sympathetic afferents along with vagal afferents provide respiratory sensations (215). Sympathetic afferents are believed to mediate the pain sensation, especially when these arising from the pleural region are involved. Patients with pleuritic pain had their symptom resolved after the stellate and upper thoracic ganglia at T2 and T3 ganglia being excised, suggesting that sympathetic afferents may be involved in generating the debilitating chest pain associated with lung abscess and tumor, pleurisy and severe pneumonitis (315). The viscerosomatic and visceravisceral convergence may be relevant to chest pain originating from trachea and lower airways, visceravisceral interactions such as cardiopulmonary reflexes, as well as the cross-organ hyperalgesia or sensitization (105), i.e. pain event originated in one organ influences the behavior of the other.

Respiratory Sensations

Respiratory sensations, such as dyspnea, air hunger, tightness of chest, airway irritation, and urge to cough, are generated

by sensory signals related to breathing or arising from the respiratory structures. The genesis of these sensations involves multiple sensors located in different parts of the respiratory systems and neural pathways, and complex signal processing in specific neural structures and regions in the CNS (37).

One of these sensors is the sensory nerve terminals located in the respiratory tract. Morton and co-workers reported the first direct evidence of respiratory sensation mediated through vagal afferents [1951]; they showed that unilateral section of the vagus nerve between recurrent laryngeal nerve and pulmonary plexus in patients with inoperable tumor in the bronchial region abolished the referred pain in the neck and chest region. Guz and co-workers later demonstrated that blocking the cervical vagus nerves with local anesthetic could attenuate the debilitating dyspnea caused by a wide variety of cardiopulmonary diseases (143). In a group of 12 patients who suffered from pulmonary vascular obstruction, lung infiltration and asthma, bilateral vagal block not only effectively reduced the dyspneic sensation, but also improved the breath-holding time and attenuated the tachypnea that was unrelated to either hypoxia or hypercapnia. Based upon the observation made in a companion experiment using the anodal block of myelinated vagal afferents in anesthetized rabbits, these investigators further suggested that pulmonary C-fibers were responsible for generating the dyspnea and tachypnea developed in these patients (143). Similar findings were later reported by other investigators in patients with various pulmonary diseases (78, 284).

Dyspnea or breathlessness is one of the most common symptoms observed in patients with various pulmonary diseases. There are different types of dypnea (144, 211), generated from a number of sensors located in different parts of the respiratory system (e.g., mechanoceptors in respiratory muscles, arterial chemoreceptors, etc) and other organs (e.g., heart). Thus, dyspnea may result from a combination of activation of different sensors under different conditions (144).

Air hunger, a desperate urge to breathe, is induced by breath holding or an increase in alveolar CO_2 concentration (19, 107), and can be relieved by increasing volume expansion of the lung (280). In high-level (C1-C2) quadriplegic patients who did not have sensory information from the chest wall or respiratory muscles, the sensation of air hunger could be increased and decreased by decreasing and increasing ventilator tidal volume, respectively, when end-tidal CO_2 was maintained at an elevated but constant level (246). Furthermore, increasing tidal volume generated less or no relief of CO_2-induced air hunger in the patients with bilateral lung transplant, who had no or diminished afferent activity arising from SARs in the lung (103, 153). All these findings suggested that stimulation of SARs by increasing inspired volume can relieve the air hunger induced by hypercapnia (103, 153, 246).

Respiratory sensations mediated through pulmonary C-fiber activation can be also generated under normal physiological conditions. Paintal and coworkers proposed that an increase in interstitial fluid volume resulting from elevated pulmonary arterial and capillary pressures stimulated pulmonary C-fibers and contributed to the breathless sensation during moderate and severe exercise (284). Furthermore, the sensitivity of these afferents can be enhanced by an increase in temperature during exercise (269, 317), adding further support to this hypothesis. However, other sensory receptors (e.g., afferents innervating skeletal muscles) are also activated during and after exertional exercise and may also contribute to the respiratory sensation under these conditions (115, 117).

Davies and co-workers made an interesting observation in a patient with unilateral pulmonary venous obstruction that section of the ipsilateral cervical vagus drastically attenuated the dyspneic sensations (78). Indeed, it has been shown that pulmonary C-fiber activity was significantly elevated by increasing left atrial pressure during pulmonary congestion in anesthetized dogs (64). Increases in pulmonary arterial and capillary pressures can also occur as a result of pulmonary vasoconstriction in healthy individuals exposed to the hypoxic environment at high altitude. Thus, it seems reasonable to suggest that the dry cough and dyspneic, choking sensations felt in the throat and upper chest of subjects with high altitude pulmonary edema are caused by activation of pulmonary C-fibers (284). It should be noted that stimulation of RARs by an increase in pulmonary interstitial volume has been extensively documented by Kappagoda and co-workers (186). Therefore, a possible involvement of RARs in generating the respiratory sensations under these conditions should not be overlooked (312). In addition, HTARs are also stimulated during lung edema. Their role in the sensation also needs to be explored.

Sensory fibers innervating the lung structures are also carried by sympathetic nerves. These "sympathetic afferents" may contribute to the respiratory sensations such as chest pain (see page 28).

Conclusion

Bestowed by modern technologies, recent studies have provided new insights into the receptor structure, signal transduction and diverse afferent properties. These new information further revealed that each of these major afferent types consists of a heterogeneous group of sensory nerves with a wide spectrum of electrophysiological and neurochemical properties, and multiple sensory modalities.

Despite these significant advances, many important and challenging questions remain to be answered. For example, properties and functions of the lung afferents will require further characterization by their molecular compositions, morphological structures, physiological functions and pharmacological profiles. NEBs are located in strategic positions in the airways with complex neural inputs and outputs. Although studied for decades, they are still a mystery as their afferent activity has not been recorded or verified yet. Revealing their function may lead to a new level of recognition of airway sensory receptors. Recent studies have demonstrated that the sensitivity of certain types of airway afferents and the expression of specific ion channels are upregulated under various

disease conditions. Although hypersensitivity of these sensory nerves is known to play a pivotal part in the manifestation of the debilitating symptoms in patients with airway inflammatory diseases, underlying mechanisms are still not fully understood. Furthermore, the involvement of vagal sensory nerves in the interaction between nervous and immunological systems will need to be delineated to advance our knowledge about this important pulmonary defense function. The vast majority of our knowledge described in this review has been derived from studies conducted in experimental animals, whereas distinct differences in airway responses have been well documented between different species; one such example is the sharp contrast between different species in the relative contributions of the centrally mediated cholinergic reflex and the local tachykininergic mechanism to bronchomotor responses to pulmonary C-fiber stimulation (for details see page 21). Hence, a critical and challenging issue will always be the selection of more suitable animal models for studying various airway diseases in humans. A continuing growth of our knowledge about the physiological and pharmacological properties of these sensory nerves will undoubtedly be essential for developing the translational research and therapeutic strategies for treating patients with airway diseases.

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A synergistic effect of simultaneous TRPA1 and TRPV1 activations on vagal pulmonary C-fiber afferents

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A synergistic effect of simultaneous TRPA1 and TRPV1 activations on vagal pulmonary C-fiber afferents

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Lin YJ, Lin RL, Ruan T, Khosravi M, Lee LY. A synergistic effect of simultaneous TRPA1 and TRPV1 activations on vagal pulmonary C-fiber afferents. *J Appl Physiol* 118: 273–281, 2015. First published November 20, 2014; doi:10.1152/japplphysiol.00805.2014.—Transient receptor potential ankyrin type 1 (TRPA1) and vanilloid type 1 (TRPV1) receptors are coexpressed in vagal pulmonary C-fiber sensory nerves. Because both these receptors are sensitive to a number of endogenous inflammatory mediators, it is conceivable that they can be activated simultaneously during airway inflammation. This study aimed to determine whether there is an interaction between these two polymodal transducers upon simultaneous activation, and how it modulates the activity of vagal pulmonary C-fiber sensory nerves. In anesthetized, spontaneously breathing rats, the reflex-mediated apneic response to intravenous injection of a combined dose of allyl isothiocyanate (AITC, a TRPA1 activator) and capsaicin (Cap, a TRPV1 activator) was ~202% greater than the mathematical sum of the responses to AITC and Cap when they were administered individually. Similar results were also observed in anesthetized mice. In addition, the synergistic effect was clearly demonstrated when the afferent activity of single vagal pulmonary C-fiber afferents were recorded in anesthetized, artificially ventilated rats; C-fiber responses to AITC, Cap and AITC + Cap (in combination) were 0.6 ± 0.1, 0.8 ± 0.1, and 4.8 ± 0.6 impulses/s (n = 24), respectively. This synergism was absent when either AITC or Cap was replaced by other chemical activators of pulmonary C-fiber afferents. The pronounced potentiating effect was further demonstrated in isolated vagal pulmonary sensory neurons using the Ca^{2+} imaging technique. In summary, this study showed a distinct positive interaction between TRPA1 and TRPV1 when they were activated simultaneously in pulmonary C-fiber sensory nerves.

inflammation; TRPA1; TRPV1; AITC; capsaicin

BOTH transient receptor potential ankyrin type 1 (TRPA1) and vanilloid type 1 (TRPV1) receptors are tetrameric membrane proteins with four identical subunits forming a ligand-gated nonselective cation channel (29, 42). In addition to having a similar protein structure as members of the superfamily of TRP channels, both channels are known to play important roles in the inflammation-induced hyperalgesia in various somatic and visceral organs (4, 5, 37, 39). In situ hybridization and immunofluorescence studies have shown that TRPA1 and TRPV1 are colocalized in dorsal root ganglion (DRG) nociceptive neurons (37). Furthermore, electrophysiological studies have demonstrated that these two channels may interact with each other in the same neurons; for example, TRPA1 is desensitized by capsaicin, a selective TRPV1 agonist, via Ca^{2+}-dependent pathway in sensory neurons (2). On the other hand, a potentiating effect of TRPA1 activation on the TRPV1 sensitivity has also been reported in DRG neurons and heterologously transfected cells (35).

In the respiratory tract, TRPA1 is localized in a subset of TRPV1-expressing vagal sensory neurons (27), similar to their distribution pattern in DRG neurons. Both channels are abundantly expressed in the vagal bronchopulmonary C-fiber sensory nerves (27) that are known to play an important role in the regulation of airway functions in normal and pathophysiological conditions (8, 22). Since both TRPA1 and TRPV1 can be activated and sensitized by a number of inflammatory mediators endogenously released in the airways, such as proton, reactive oxygen species, prostanoids, and bradykinin (4, 6, 23, 32, 39, 44), it is conceivable that they can be activated simultaneously during airway inflammatory reaction. Hence, this study was carried out to investigate whether there is an interaction between TRPA1 and TRPV1 upon simultaneous activation, and how this interaction may modulate the activity of vagal pulmonary C-fiber sensory nerves.

METHODS AND MATERIALS

This study comprised two parts: in vivo and in vitro studies. The experimental procedures described below were in accordance with the recommendation in *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health and also approved by the University of Kentucky Institutional Animal Care and Use Committee.

In Vivo Study

Animal preparation. The experiments were carried out in two rodent species: Sprague-Dawley rats (352.9 ± 8.3 g, n = 55) and C57BL6/J mice (27.3 ± 1.8 g, n = 6). Animals were initially anesthetized with an intraperitoneal injection of α-chloralose (rat: 100 mg/kg; mouse: 70 mg/kg) and urethane (rat: 500 mg/kg; mouse: 1,000 mg/kg) dissolved in a 2% borax solution; supplemental doses (one-tenth of the initial dose) of the same anesthetics were injected intravenously to maintain abolition of pain reflexes induced by tail-pinch. For administration of pharmacological agent(s), a catheter was inserted into the left jugular vein and advanced until its tip was positioned just above the right atrium. A catheter was inserted into femoral artery and connected to a pressure transducer (Statham P23AC, Hato Rey, Puerto Rico) for recording the arterial blood pressure (ABP) and heart rate (HR). A short tracheal cannula was inserted just below the larynx via a tracheotomy. Body temperature was maintained at ~36°C by means of heating pad placed under the animal lying in a supine position. At the end of experiment, animals were euthanized by intravenous injection of 3M KCl (2 ml for rats and 0.2 ml for mice).

Study 1: pulmonary chemoreflex response. Animals breathed spontaneously through the tracheal cannula. Respiratory flow was measured by a heated pneumotachograph (University of Kentucky Center

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for Manufacturing) connected to a differential pressure transducer (MP45-14; Validyne, Northridge, CA), and integrated by an integrator to give tidal volume (V_T). Pulmonary chemoreflex responses were measured in each animal when intravenous injections of allyl isothiocyanate (AITC, a selective TRPA1 agonist, 0.8–1.2 mg/kg in rats and 0.3–0.6 mg/kg in mice) and capsaicin (Cap, a selective TRPV1 agonist, 0.75 μg/kg in rats and 0.25–0.50 μg/kg in mice) were administered individually first, and then in combination in anesthetized, spontaneously breathing rats ($n = 6$) and mice ($n = 6$); 20 min was allowed to elapse between two consecutive injections to avoid tachyphylaxis.

Respiratory frequency (f), V_T, and expiratory duration (T_E) were analyzed on a breath-by-breath basis using a data-acquisition system (TS-100; Biocybernetics, Taipei, Taiwan). To determine the intensity of apneic response, the apneic ratio was calculated by dividing the longest T_E occurring within the first 5 breaths after the injection by the baseline T_E that was averaged over 20 breaths immediately preceding the injection.

Study 2: pulmonary C-fiber activity. Single-unit activities of vagal pulmonary C-fibers were recorded from anesthetized, open-chest, and artificially ventilated rats. V_T and f were set at 8 ml/kg and 50 breaths/min (7025; UGO Basile, Comerio-Varese, Italy), respectively, to mimic those of anesthetized, unilaterally vagotomized rats. The right cervical vagus nerve was sectioned, and its caudal end was placed on a small dissecting platform and immersed in a pool of mineral oil. A thin filament was teased away from the desheathed nerve trunk and placed on a platinum-iridium hook electrode. Action potentials were amplified by a preamplifier (P511K; Grass Technologies, Warwick, RI), and monitored by an audio monitor (AM8RS; Grass Technologies). The thin filament was further split until the afferent activity arising from a single unit was electrically isolated. The afferent activity of a single unit was first searched by hyperinflation (3–4 times V_T) and then identified by the immediate (delay $<$ 1 s) response to an intravenous bolus injection of a larger dose of Cap (1 μg/kg). At the end of the experiment, the general locations of pulmonary C-fiber endings were identified by their responses to the gentle pressing of the lungs with a blunt-ended glass rod. A total of 65 C-fibers were studied in 49 rats.

Pulmonary C-fiber activities were recorded when intravenous injections of AITC (0.50–0.75 mg/kg) and Cap (0.35–0.75 μg/kg) were administered individually first, and then in combination; 20 min elapsed between two injections. In a separate study series, an identical protocol was followed when AITC was replaced by cinnamaldehyde (1.5–2.0 mg/kg), another selective activator of TRPA1. To investigate if the C-fiber responses were uniquely generated by the interaction between TRPA1 and TRPV1 activations, we replaced either AITC or Cap by one of the three known chemical stimulants of C-fibers: phenylbiguanide (PBG, 2–4 μg/kg), a 5-HT_3 receptor agonist (25); adenosine 5′-triphosphate (ATP, 0.6–2.0 mg/kg), an agonist of P2X2 and P2X3 receptors (19); and adenosine (Ado, 0.02–0.60 mg/kg), an A_1 adenosine receptor agonist (16). To avoid the stimulation of pulmonary C-fibers generated by a larger volume of bolus injection, a combined dose of AITC and Cap was injected in the same volume as that for the single injections.

Fiber activity (FA) was continuously recorded and analyzed for 20 s before and 60 s after each injection. Baseline FA was averaged over the 10-s period immediately preceding the injection, and the peak response was defined as the maximum 5-s average FA within the first 10 s after the injection.

In Vitro Study

Fluorescent labeling and isolation of vagal pulmonary sensory neurons. Sensory neurons innervating the lungs and airways were identified by retrograde labeling from the lungs by using the fluorescent tracer 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Sigma, St. Louis, MO) (20). Young Sprague-Dawley rats (130–180 g) were anesthetized by inhalation of vaporized isoflurane (2% in O_2). After a small midline incision was made on the ventral neck skin, DiI (0.2 mg/ml; 0.05 ml) was instilled into the trachea and lung via a needle (28 gauge). The incision was then closed by tissue adhesive (Vetbond; 3M, St. Paul, MN). Seven to ten days later, DiI-pretreated animals were anesthetized and decapitated. The head was immediately immersed in ice-cold DMEM/F-12 solution followed by quick extraction of nodose and jugular ganglia. Each ganglion was then desheathed, cut, and placed in a mixture of type IV collagenase (0.04%) and dispase II (0.02%), and incubated for 80 min in 5% CO_2 in air at 37°C. After digestion, centrifugation and resuspension, cells were dissociated by gentle trituration with small-bore, fire-polished Pasteur pipettes. Myelin debris was discarded after centrifugation of the dispersed cell suspension (500 *g*, 8 min). The cell pellets were resuspended in the modified DMEM/F-12 solution (20),

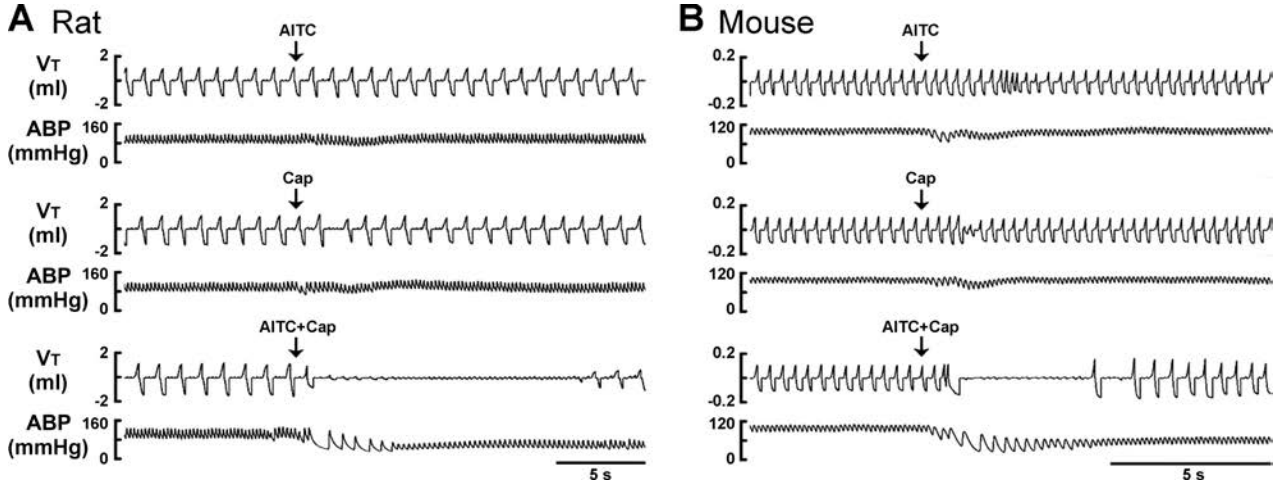

Fig. 1. Experimental records illustrating the synergistic effects of transient receptor potential ankyrin type 1 (TRPA1) and vanilloid type 1 (TRPV1) agonists on pulmonary chemoreflex in spontaneously breathing rat (*A*) and mouse (*B*). Arrows depict intravenous bolus injections of allyl isothiocyanate (AITC, a TRPA1 agonist; 0.8 mg/kg for rat, 0.6 mg/kg for mouse; top panels), capsaicin (Cap, a TRPV1 agonist; 0.75 μg/kg for rat, 0.5 μg/kg for mouse; middle panels), and a combination of AITC and Cap with each at the same dose as that administered alone (bottom panels). The same volume of chemical solutions (0.15 ml in rat and 0.05 ml in mouse) was delivered in each injection regardless whether a single chemical or two chemicals in combination were given. The interval between injections was 20 min. V_T, tidal volume; ABP, arterial blood pressure. Body weights of the rat and the mouse were 374 g and 28.1 g, respectively.

plated onto poly-L-lysine-coated glass coverslips, and then incubated overnight (5% CO_2 in air at 37°C).

Study 3: measurement of Ca^{2+} transient in rat pulmonary sensory neurons. Cultured cells (as described above) were washed and maintained in an extracellular solution (ECS). Ca^{2+} transients were measured in these cells with a digital fluorescence microscope (Axiovert 100; Carl Zeiss, Thornwood, NY) equipped with a variable filter wheel (Sutter Instruments, Novato, CA) and a digital CCD camera (Princeton Instruments, Trenton, NJ) (15). Cells were incubated with 5 μM Fura-2 AM (Life Technologies, Grand Island, NY), a Ca^{2+} indicator, for 30 min at 37°C, rinsed (×3) with ECS, and then allowed to deesterify for 30 min before use. Dual images (340- and 380-nm excitation, 510-nm emission) were collected, and pseudocolor ratiometric images were monitored during the experiments by using the software Axon Imaging Workbench (Axon Instruments, Union City, CA).

A total of 156 DiI-labeled neurons from six rats were studied. The coverslip containing the cells was mounted into a chamber continuously perfused with an ECS during the experiment by a gravity-fed valve-controlled system (VC-66CS, Warner Instruments, Hamden, CT) at a constant rate of ~2 ml/min. The KCl solution (60 mM, 20 s) was perfused at the end of each experimental run to determine the cell viability. Ca^{2+} transients were recorded and compared when AITC (150 μM, 30 s) and Cap (100–150 nM, 30 s) were administered individually first, and then in combination; >15 min elapsed between two consecutive challenges. The order of administration was reversed in 109 neurons. The Fura-2 fluorescence 340/380 ratio was continuously analyzed at 2-s intervals, and the Ca^{2+} transient was measured as increase in the 340/380 ratio (ΔRatio): the difference between the peak amplitude (4-s average) after the challenge and the baseline (30-s average).

Statistical Analysis

In both in vivo and in vitro studies, data were compared using the one-way analysis of variance (ANOVA). A value of $P < 0.05$ was considered significant. All data are reported as means ± SE.

Chemical Agents

In the in vivo study, stock solutions of Cap (250 μg/ml), AITC (50 mg/ml), and cinnamonaldehyde (50 mg/ml) were prepared in 10% Tween 80, 10% ethanol, and 80% saline, and that of ATP (20 mg/ml), Ado (10 mg/ml), and PBG (1 mg/ml) were prepared in saline. These stock solutions were stored at −20°C, and prepared daily at the desired concentrations for injection by dilution with isotonic saline based on the animal's body weight. In the in vitro study, desired concentrations of the chemical agents were prepared in a similar manner, except that the ECS, instead of saline, was used as the

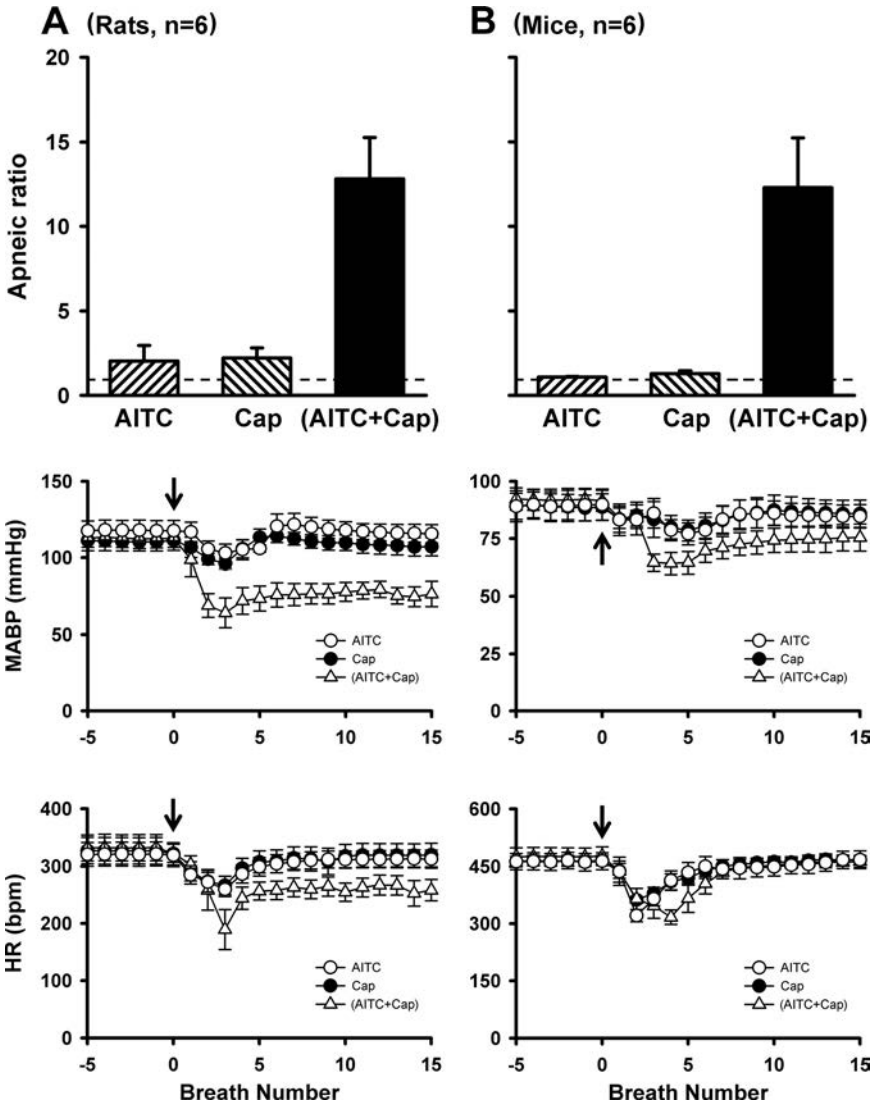

Fig. 2. Synergistic effects of TRPA1 and TRPV1 agonists on pulmonary chemoreflex responses in rats and mice. AITC dose: 0.8–1.2 mg/kg for rats and 0.3–0.6 mg/kg for mice; Cap dose: 0.75 μg/kg for rats and 0.25–0.50 μg/kg for mice. "AITC + Cap," responses to a combination of AITC and Cap with each at the same dose as that administered alone in the same animal. The volume injected (0.15 ml for rats and 0.05 ml for mice) was the same in all injections. Top panels: the apneic ratio was calculated as maximum expiratory duration (T_E) occurring during the first 5 breaths after injection divided by average of 20 breaths of the baseline T_E. Dashed line represents apneic ratio of 100% level (no apnea). Middle and bottom panels: responses of mean arterial blood pressure (MABP) and heart rate (HR) to injections of AITC alone, Cap alone, and a combination of AITC and Cap. Arrows depict the time of injections. Data are means ± SE ($n = 6$ for both rats and mice).

diluent. The ECS contained (in mM) 5.4 KCl, 136 NaCl, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 0.33 NaH_2PO_4, 10 glucose, 10 HEPES, and a pH level adjusted to 7.4 with NaOH and the osmolarity to 300 mOsm. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except dispase II (Roche, Indianapolis, IN), DMEM/F12 (Invitrogen, Carlsbad, CA), and Fura-2 AM (Life Technologies, Grand Island, NY).

RESULTS

Study 1

To avoid nonselective activation of TRPA1 and TRPV1 receptors (11, 13), only low (slightly above threshold) doses of AITC (0.8–1.2 mg/kg for rats and 0.3–0.6 mg/kg for mice) and Cap (0.75 μg/kg for rats and 0.25–0.50 μg/kg for mice) were used, respectively, in this study. Figure 1*A* shows representative tracings of acute respiratory and cardiovascular responses to intravenous injections of AITC, Cap, and a combination of AITC and Cap in an anesthetized, spontaneously breathing rat. Injection of AITC or Cap immediately (within 1–3 breaths) evoked a modest apneic response. In a sharp contrast, the apneic response to the injection of a combination of the same doses of AITC and Cap was markedly potentiated, accompanied by intense bradycardia and hypotension. The group data showed that the apneic ratios triggered by injections of AITC alone and Cap alone were 2.0 ± 0.9 and 2.2 ± 0.6, respectively, whereas a combined injection of AITC and Cap at the same doses elevated the apneic ratio to 12.8 ± 2.4 ($P < 0.05$, $n = 6$; Fig. 2*A*), which was 202% greater than the mathematical sum of TRPA1 and TRPV1 responses (Fig. 2*A*). This synergistic effect was also clearly demonstrated in mice (Fig. 1*B*); the apneic response to intravenous injection of AITC and Cap in combination was 425% greater than the mathematical sum of the responses to the same doses of AITC and Cap when they were injected individually ($P < 0.05$, $n = 6$; Fig. 2*B*).

Study 2

Intravenous injections of low doses of AITC (0.50–0.75 mg/kg) and Cap (0.35–0.75 μg/kg) immediately evoked pulmonary C-fiber discharge (e.g., Fig. 3*A*, left and middle panels); in comparison, the latency of the C-fiber response to AITC (2–3 s) was slightly, but consistently, longer than that of Cap (~1 s). When the same doses of AITC and Cap were injected in combination, the afferent activity was strikingly amplified (Fig. 3*A*, right panel), accompanied by pronounced bradycardia and hypotension; no potentiation was found in only 5 of the 24 C-fibers studied. Group data showed that the peak fiber activity evoked by an injection of AITC and Cap in combination (4.8 ± 0.6 impulses/s; $n = 24$) was significantly greater than that evoked by AITC alone (0.6 ± 0.1 impulses/s; $P < 0.05$), Cap alone (0.8 ± 0.1 impulses/s; $P < 0.05$), or the mathematical sum of the two (1.4 ± 0.2 impulses/s; $P < 0.05$) (Fig. 4*B*). Notably, the duration of fiber discharge was also markedly prolonged by the combined injection of AITC and Cap (Fig. 4*A*): 2.24 ± 0.28, 1.82 ± 0.15, and 5.65 ± 0.74 s after injections of AITC alone, Cap alone, and a combination of AITC and Cap, respectively ($P < 0.05$). This strong potentiation was also found when cinnamaldehyde, another selective agonist of TRPA1, was administered in combination with Cap (Fig. 3*B*), which clearly amplified both the peak activity and duration of the C-fiber discharge (Fig. 4, *C* and *D*).

To test the possibility that certain unknown chemical product(s) was formed after AITC and Cap were mixed in solution

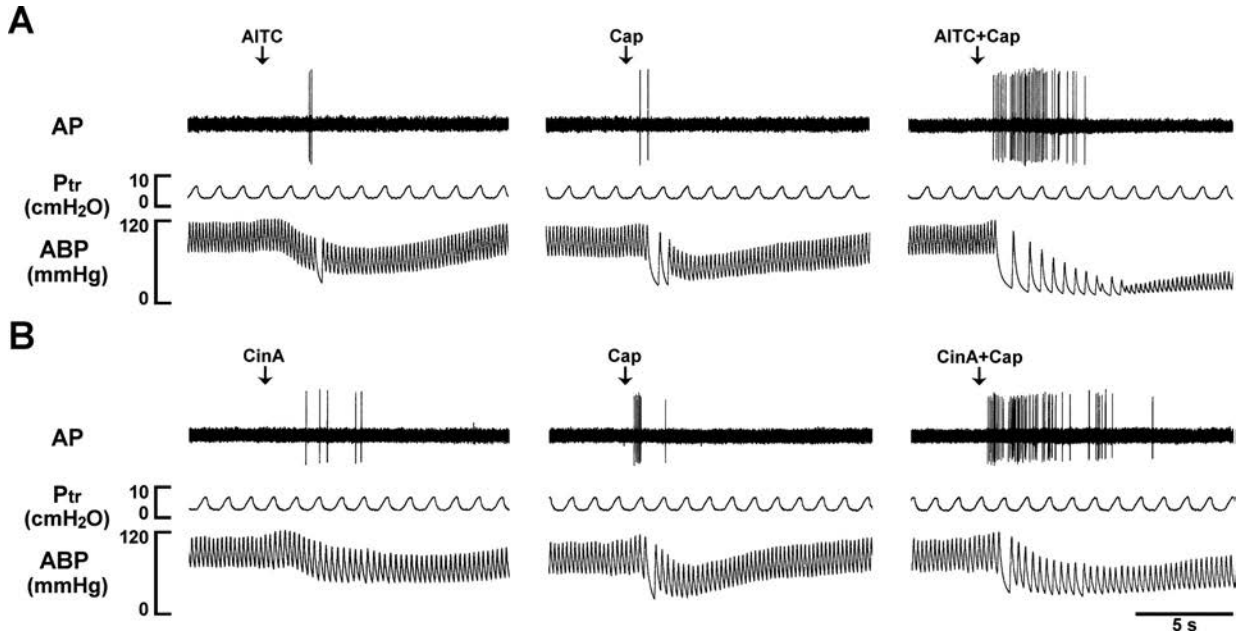

Fig. 3. Experimental records illustrating the synergistic effects of TRPA1 and TRPV1 agonists on pulmonary C-fibers in anesthetized and artificially ventilated rats. *A*: responses of pulmonary C-fiber arising from the lower lobe of right lung to intravenously injections of AITC (0.75 mg/kg; left panel), Cap (0.75 μg/kg; middle panel), and a combination of AITC and Cap at the same doses (right panel). *B*: responses of pulmonary C-fiber arising from the lower lobe of right lung to intravenously bolus injections of cinnamaldehyde (CinA, 1.8 mg/kg; left panel), Cap (0.50 μg/kg; middle panel), and a combination of CinA and Cap at the same doses (right panel). The same volume (0.15 ml) was delivered in all injections, first into the catheter (dead space volume 0.2 ml) and then flushed (at arrow) into the circulation by a bolus of 0.3 ml saline. The interval between injections was 20 min. Rat body weights for *A* and *B* were 310 and 280 g, respectively. AP, action potential; P_{tr}, tracheal pressure; ABP, arterial blood pressure.

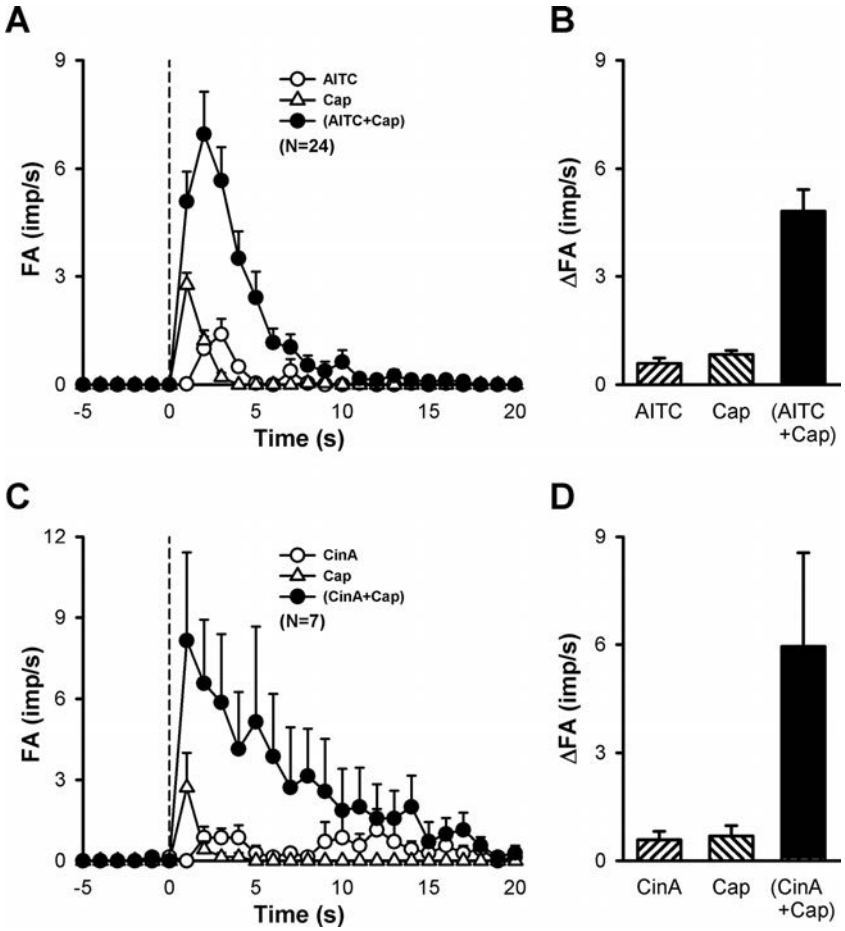

Fig. 4. Synergistic effects of TRPA1 and TRPV1 agonists on pulmonary C-fiber afferent activity in anesthetized rats. *A*: histograms of pulmonary C-fibers activity (FA) to intravenous injections (dashed line) of AITC (0.50–0.75 mg/kg), Cap (0.35–0.75 μg/kg), and a combination of AITC and Cap (each at the same dose as that injected separately in the same fiber). *B*: data in *A* are converted to ΔFA, which is calculated as the difference between the peak FA (5-s average) and the 10-s average of the baseline FA in each fiber. Descriptions of *C and D* are similar to that of *A* and *B*, except that AITC was replaced by cinnamaldehyde (CinA, 1.5–2.0 mg/kg). Data are means ± SE. Imp/s, impulses per second.

and contributed to the potentiating effect, we injected AITC and Cap simultaneously but separately into right and left jugular venous catheters, respectively. The pronounced amplification of the response was similar to that elicited by one bolus injection of the combined solution (Fig. 5).

To determine if simultaneous activations of both TRPA1 and TRPV1 are required in generating this synergistic effect, we studied the C-fiber responses when either AITC (Fig. 6) or Cap (Fig. 7) was replaced by another known chemical activator of pulmonary C-fibers, such as PBG, ATP, and Ado. In distinct contrast, the average peak fiber activity evoked by the combined injection was not higher than the mathematical sum of two individual injections in any of these combinations (Figs. 6 and 7).

Study 3

Application of a low concentration of AITC or Cap alone evoked a mild and transient increase in the intracellular Ca^{2+} concentration (measured by the Fura-2 340/380 ratio) in isolated rat vagal pulmonary sensory neurons, whereas the Ca^{2+} transients were markedly elevated by a combined challenge of

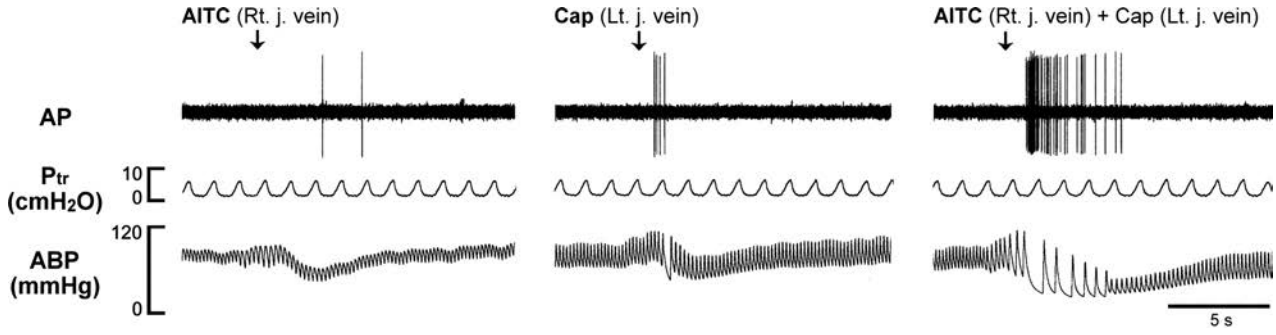

Fig. 5. Experimental records illustrating the synergistic effects of AITC and Cap on a pulmonary C-fiber when they were injected via different venous catheters in an anesthetized and artificially ventilated rat. AITC (0.6 mg/kg, left panel) and Cap (0.5 μg/kg, middle panel) were injected via the catheters inserted in right and left jugular veins, respectively; the same doses of AITC and Cap were injected separately but simultaneously (right panel). The interval between injections was 20 min. Rt. j., right jugular; Lt. j., left jugular. Rat body weight: 342 g. For detailed explanations, see legend of Fig. 3.

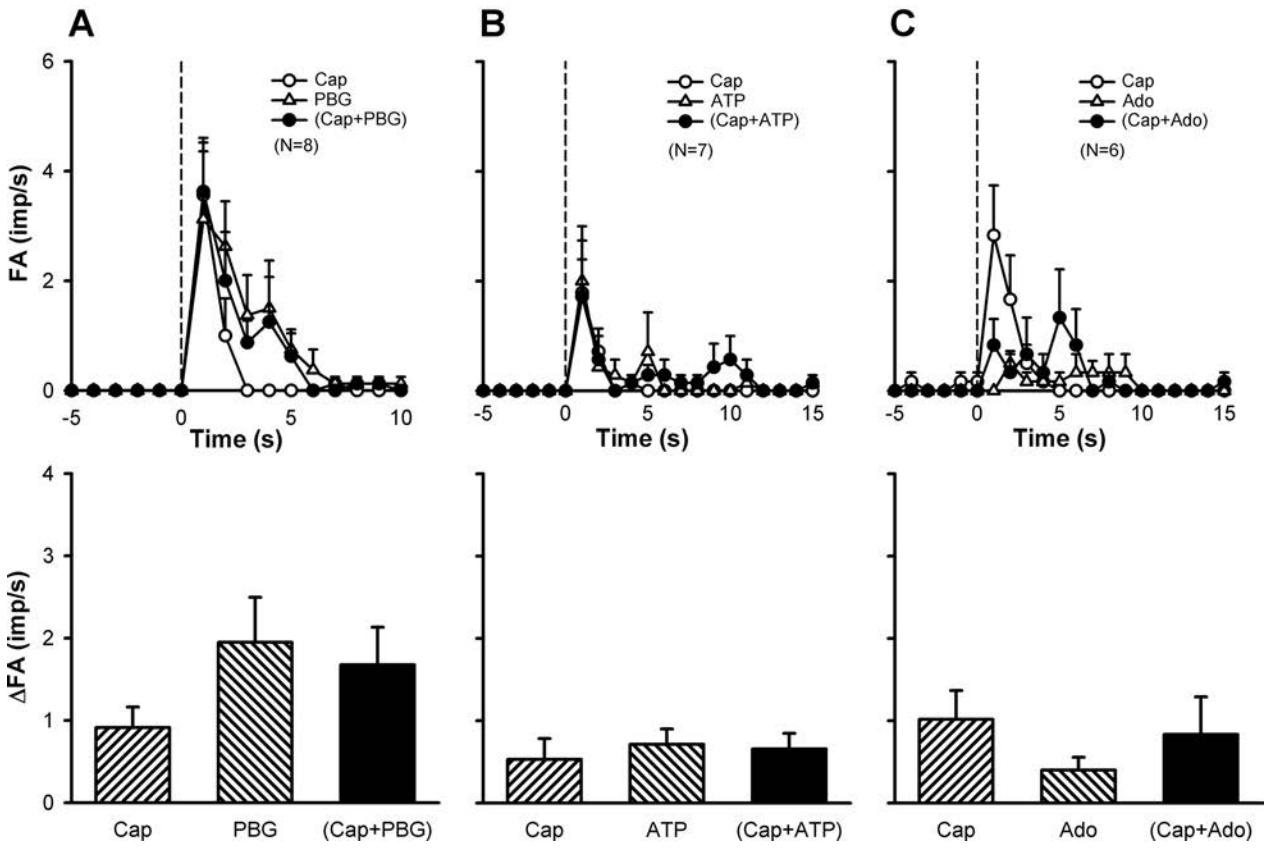

Fig. 6. A lack of synergistic effect when the TRPA1 agonist was replaced by other known C-fiber activators. The TRPA1 agonist was replaced by phenylbiguanide (PBG; 2.0–4.0 μg/kg; *A*), ATP (0.75–1.0 mg/kg; *B*), or adenosine (Ado; 0.02–0.05 mg/kg; *C*). Dose of capsaicin (Cap) was 0.20–0.75 μg/kg. For detailed explanations, see the legend of Fig. 4.

AITC and Cap in the same neurons (e.g., Fig. 8*A*). This potentiation was also found when the order of administration was reversed (e.g., Fig. 8*B*). Group data indicated that the response, as measured by Δ(340/380 ratio), evoked by a combined challenge of AITC and Cap, was significantly greater than the mathematical sum of the responses to the same concentrations of AITC and Cap when they were administered individually ($P < 0.05$, $n = 156$; Fig. 8*C*).

DISCUSSION

Both TRPA1 and TRPV1 are polymodal transducers abundantly expressed in vagal bronchopulmonary C-fiber sensory nerves (22, 27, 45). Both these TRP channels can be activated by a number of endogenous chemical mediators, for example, TRPV1 by hydrogen ion, anandamide and *N*-arachidonyl dopamine (14, 18), and TRPA1 by 4-hydroxynonenal, 4-oxononenal, bradykinin, and reactive oxygen species (e.g., hydrogen peroxide, nitric oxide, etc.) (3, 5, 7, 24, 38). Furthermore, the sensitivity of these receptors can be enhanced by certain endogenous inflammatory mediators, such as prostaglandin E_2 and bradykinin (14, 18, 20). It is, therefore, highly possible that both TRPA1 and TRPV1 can be activated simultaneously during airway inflammatory reaction.

This study demonstrated that a simultaneous activation of both TRPA1 and TRPV1 receptors generated a distinct synergistic effect on vagal pulmonary C-fiber sensory nerves, which was manifested by a pronounced potentiation of the pulmonary chemoreflex responses. A similar synergistic effect was also observed in isolated vagal pulmonary sensory neurons, indicating that the positive interaction between TRPA1 and TRPV1 occurs primarily at the sensory neurons. It appears that this interaction occurs specifically between TRPA1 and TRPV1 because the synergy was completely absent when the selective agonist of one of these channels was replaced by other chemical activators of pulmonary C-fibers. This striking synergistic effect is not species-dependent because a similar effect was also observed in the pulmonary chemoreflex responses in mice (Fig. 1).

In this study, low doses of AITC and Cap were chosen for selectively activating TRPA1 and TRPV1, respectively, because recent studies reported that AITC at a higher concentration (3 mM) can activate TRPV1 in DRG and transfected cells (11, 13). Indeed, both AITC and Cap delivered alone at these low doses induced only very modest responses in pulmonary chemoreflexes, C-fiber discharges, and Ca^{2+} transients in this study. In a striking contrast, distinctly amplified responses were evoked when the same low doses of AITC and Cap were delivered in combination.

We questioned if the synergistic effect was generated by certain unknown chemical product(s) formed when AITC (or cinnamaldehyde) and Cap were mixed in solution before injection. This possibility can be ruled out because the potentiated responses persisted when these two chemical agents were injected simultaneously, but via two separate routes (Fig. 5).

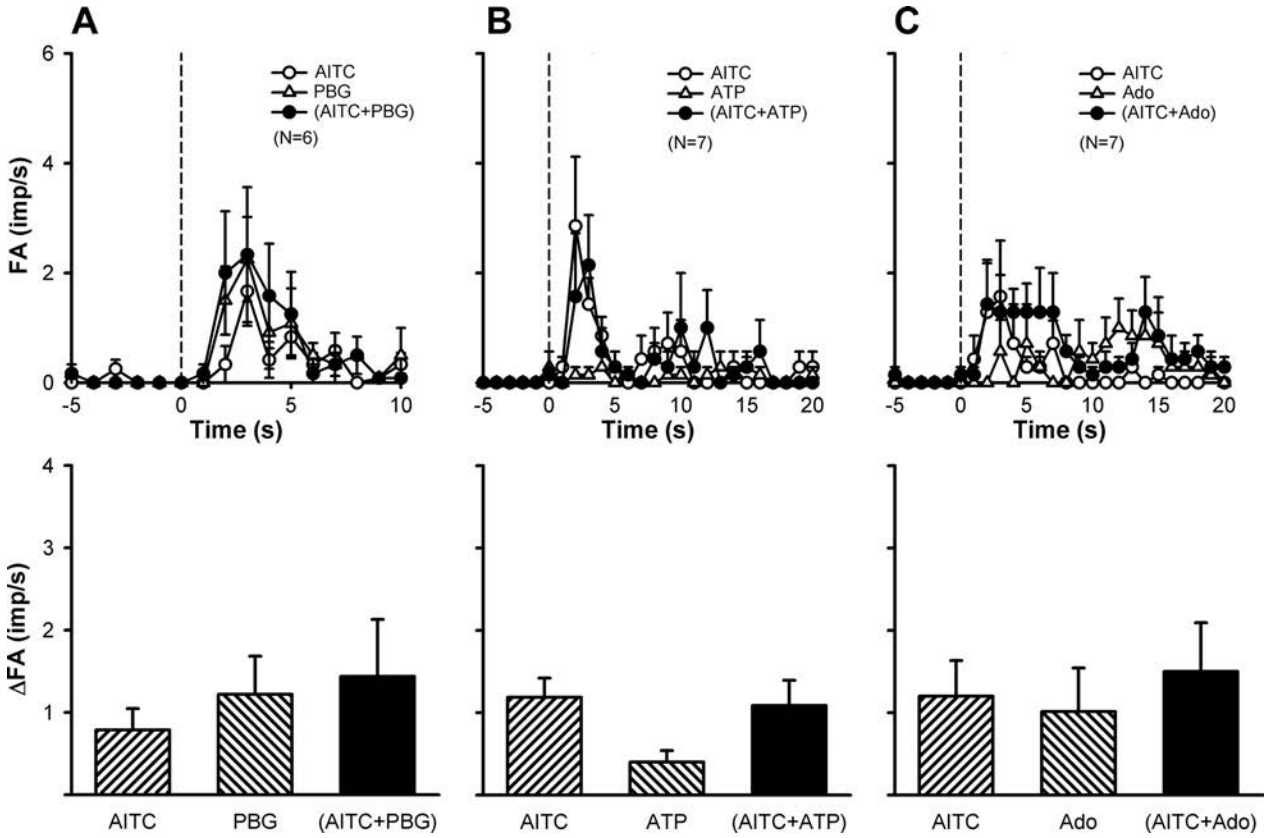

Fig. 7. A lack of synergistic effect when the TRPV1 agonist was replaced by other known C-fiber activators. The TRPV1 agonist was replaced by PBG (2.0–4.0 μg/kg; *A*), ATP (0.6–2.0 mg/kg; *B*) or Ado (0.15–0.60 mg/kg; *C*). Dose of AITC was 0.25–1.0 mg/kg. For detailed explanations, see the legend of Fig. 4.

Results of this study suggest that a functional linkage between TRPA1 and TRPV1 channels in these sensory neurons is probably an important contributing factor. However, the underlying mechanism(s) of this synergistic effect is not yet known, and the relative roles and contributions of TRPA1 and TRPV1 also remain to be determined. When AITC and Cap were administered in combination, did it enhance the current through TRPA1, TRPV1, both these channels, or some yet unidentified channel(s)? The elevated responses of these channels may involve either or both of the two possible mechanisms: increases in receptor expression and channel sensitivity. Schmidt et al. (34) indicated that application of mustard oil (AITC) to TRPA1-transfected cells triggered the TRPA1 trafficking to the plasma membrane. They also found that application of Cap raised cell surface TRPA1 protein expression, but not that of TRPV1 in the TRPA1/TRPV1-coexpressing

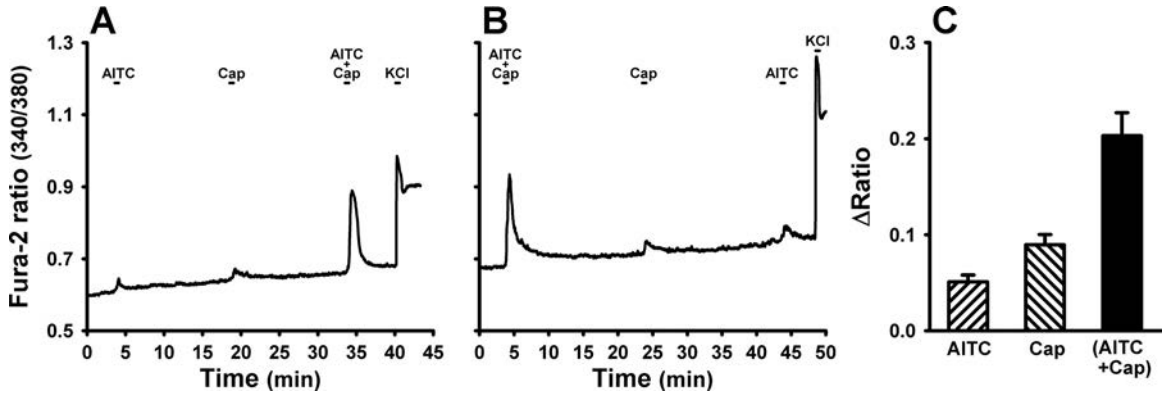

Fig. 8. Changes in intracellular Ca^{2+} concentration evoked by TRPA1 and TRPV1 agonists in isolated rat pulmonary sensory neuron(s). *A*: experimental record illustrating responses to AITC (150 μM for 30 s), Cap (100 nM for 30 s), and a combination of AITC and Cap in a pulmonary jugular sensory neuron. KCl (60 mM for 20 s) was applied to test the cell vitality at the end of the experiment. *B*: experimental record illustrating responses to a reversed sequence of challenges in a different pulmonary jugular sensory neuron; the concentrations of AITC and Cap were the same as that in *A*. *C*: averaged group data (156 DiI-labeled cells). The intracellular Ca^{2+} concentration was measured as the Fura-2 fluorescence 340/380 ratio, and ΔRatio was calculated as the difference between peak ratio (averaged over 4-s interval) and baseline ratio (average over 30-s interval); "AITC + Cap" was the response to a combination of AITC and Cap with each at the same concentration as that was administered separately. Data are means ± SE.

cells. It has been reported that a translocation of TRPV1 from intracellular pool to plasma membrane led to TRPV1 sensitization (40, 43). However, in those studies described above, a much longer duration (5–30 min) of pretreatment with receptor agonists was required for the trafficking and translocation of these proteins to take place. In comparison, in our study the potentiating effect was immediately (within 1–2 s) evoked by a combined dose of TRPA1 and TRPV1 agonists. Thus we do not believe that the change in receptor expression and translocation is a plausible explanation for the synergistic effect observed in our study.

Another mechanism possibly involved in this potentiating effect that merits further consideration is an enhanced sensitivity of TRPA1 and/or TRPV1. Cumulative evidence suggests that the intracellular second messengers, such as Ca^{2+} or cyclic adenosine monophosphate (cAMP), may participate in the TRPA1-TRPV1 interaction. Intracellular Ca^{2+} can activate TRPA1 by binding the EF-hand domain in its NH_2 terminus (10, 46). In addition, increased intracellular Ca^{2+} might also modulate the protein kinases (PKs), such as PKC and PKA, which further resulted in phosphorylation and sensitization of TRPV1 (17, 30, 41). Activation of TRPV1 can trigger more Ca^{2+} influx, which may in turn lead to TRPA1 activation (1, 4). On the other hand, a recent study showed that TRPA1 activation induced Ca^{2+} influx and elevated cAMP levels, which then activated PKA and led to TRPV1 sensitization (35).

Staruschenko and coworkers (36) and Salas and coworkers (33) recently reported that functional heteromeric channels can be formed from TRPA1 and TRPV1 on the cytoplasmic membrane, and that TRPV1 can influence intrinsic characteristics of the TRPA1 channel, probably through direct interaction of the channels within the complex, independent of the change of intracellular Ca^{2+}. Whether and to what extent the TRPA1-TRPV1 interaction occurring in the heteromeric TRPA1-TRPV1 complexes contributes to the synergistic effect observed in our study requires further investigation.

It is well documented that TRPA1 and TRPV1 are also expressed in the nonneuronal cells (12), although the levels of protein expression, particularly for TRPV1, are considerably lower in other cell types. TRPA1 expression was found in airway epithelial cells, fibroblasts, lymphocytes, etc.; and the TRPV1 expression detected in bronchial epithelial cells (26). Thus it is conceivable that activation of TRPA1 may have triggered the release of intermediate mediator(s) from other target cells in the airways and pulmonary vessels, which in turn led to the sensitization of C-fiber afferents to Cap. Indeed, recent studies have shown that TRPA1 or TRPV1 activation on bronchial epithelial cells promoted proinflammatory mediator secretion (28, 31). However, the fact that onset of this synergistic effect arose so rapidly (1–2 s) after the injection seems to argue against such a possibility. Furthermore, this potentiating effect was also found in isolated pulmonary sensory neurons based upon our Ca^{2+} data, suggesting that the positive interaction occurs more likely at the sensory nerves.

The C-fiber sensory nerve endings expressing TRPA1 and TRPV1 are extensively found in the airway mucosa as well as in the deeper layer of airway tissue (45). Upon activation, they elicit centrally mediated reflex responses, which include bronchoconstriction and mucus hypersecretion via the cholinergic pathway, accompanied by the sensation of airway irritation and urge to cough (8, 22). Activation of these sensory nerves can also trigger release of tachykinins and calcitonin gene-related peptide (CGRP) from the sensory terminals, which can act on a number of effector cells in the respiratory tract (e.g., smooth muscles, cholinergic ganglia, mucous glands, immune cells), and elicit the local "axon reflexes" such as bronchoconstriction, protein extravasation, and inflammatory cell chemotaxis (9, 21). Because both TRPA1 and TRPV1 are sensitive to a number of endogenous inflammatory mediators (3, 5, 7, 14, 18, 24, 38), it is probable that they can be activated simultaneously during acute or chronic airway inflammatory reaction. The synergistic effect of activating these two channels will amplify the intensity of bronchopulmonary C-fiber discharge as demonstrated in this study, which may lead to the manifestation of various symptoms of airway hypersensitivity. Whether this effect occurs in patients with airway inflammatory diseases remains to be determined.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: Y.-J.L., R.-L.L., M.K., and L.-Y.L. conception and design of research; Y.-J.L., R.-L.L., T.R., and L.-Y.L. performed experiments; Y.-J.L., R.-L.L., and L.-Y.L. analyzed data; Y.-J.L., R.-L.L., T.R., M.K., and L.-Y.L. interpreted results of experiments; Y.-J.L., R.-L.L., and L.-Y.L. prepared figures; Y.-J.L., R.-L.L., M.K., and L.-Y.L. drafted manuscript; Y.-J.L., R.-L.L., M.K., and L.-Y.L. edited and revised manuscript; Y.-J.L., R.-L.L., T.R., M.K., and L.-Y.L. approved final version of manuscript.

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Hemorrhagic hypotension-induced hypersensitivity of vagal pulmonary C-fibers to chemical stimulation and lung inflation in anesthetized rats

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Lin RL, Lin YJ, Xu F, Lee LY. Hemorrhagic hypotension-induced hypersensitivity of vagal pulmonary C-fibers to chemical stimulation and lung inflation in anesthetized rats. *Am J Physiol Regul Integr Comp Physiol* 308: R605–R613, 2015. First published January 14, 2015; doi:10.1152/ajpregu.00424.2014.—This study was carried out to investigate whether hemorrhagic hypotension (HH) altered the sensitivity of vagal pulmonary C-fibers. The fiber activity (FA) of single vagal pulmonary C-fiber was continuously recorded in anesthetized rats before, during, and after HH was induced by bleeding from the femoral arterial catheter into a blood reservoir and lowering the mean systemic arterial pressure (MSAP) to ~40 mmHg for 20 min. Our results showed the following. First, after MSAP reached a steady state of HH, the peak FA response to intravenous injection of capsaicin was elevated by approximately fivefold. The enhanced C-fiber sensitivity continued to increase during HH and sustained even after MSAP returned to baseline during the recovery, but slowly returned to control ~20 min later. Second, responses of FA to intravenous injections of other chemical stimulants of pulmonary C-fibers (phenylbiguanide, lactic acid, and adenosine) and a constant-pressure lung hyperinflation were all significantly potentiated by HH. Third, infusion of sodium bicarbonate alleviated the systemic acidosis during HH, and it also attenuated, but did not completely prevent, the HH-induced C-fiber hypersensitivity. In conclusion, the pulmonary C-fiber sensitivity was elevated during HH, probably caused by the endogenous release of chemical substances (e.g., lactic acid) that were produced by tissue ischemia during HH. This enhanced C-fiber sensitivity may heighten the pulmonary protective reflexes mediated through these afferents (e.g., cough, J reflex) during hemorrhage when the body is more susceptible to other hazardous insults and pathophysiological stresses.

hemorrhage; pulmonary; C-fiber; hypoxia; hypersensitivity

VAGAL BRONCHOPULMONARY C-fibers represent ~75% of the vagal afferents arising from the lung and airways (17). These unmyelinated afferents innervate the entire respiratory tract and play an important role in regulating the respiratory functions under various physiological and pathophysiological conditions (5, 21). Activation of these afferents elicits centrally mediated protective reflex responses via the cholinergic pathway, which include laryngeal closure, bronchoconstriction and airway hypersecretion, accompanied by cough, airway irritation, dyspneic sensation, and J reflex (5, 21). Activation of these sensory nerves can also trigger release of tachykinins and calcitonin gene-related peptide from the sensory terminals, which can act on a number of effector cells in the respiratory tract (e.g., smooth muscles, cholinergic ganglia, mucous glands, immune cells), and elicit the local "axon reflexes" such as bronchoconstriction, protein extravasation, and inflammatory cell chemotaxis (5, 18, 21).

Tissue ischemia is known to activate C-fiber nociceptors in various organ systems including heart, gastrointestinal tract, and skeletal muscles (2, 9, 20). However, its action on C-fibers in the lung is not known. Severe hemorrhage, a medical condition frequently encountered in patients who suffer from traumatic injury, can lead to tissue ischemia, hypoxia, and acidosis (8), accompanied by elevated levels of adenosine (32), lactate (23), pro-inflammatory cytokines (7), and reactive oxygen species (ROS) (27) in the circulating blood. Many of these chemical substances and mediators are known to exert potent stimulatory and sensitizing effects on pulmonary C-fiber afferents (21). In particular, acidosis can both activate (15) and sensitize vagal pulmonary C-fibers (10) to chemical stimuli. Hypoxia has also been shown to enhance the pulmonary C-fiber sensitivity to capsaicin (30).

In view of the background information described above, this study was carried out *1*) to determine the effects of hemorrhagic hypotension (HH) on the baseline activity and sensitivity of vagal pulmonary C-fiber afferents to chemical and mechanical stimuli; and *2*) to identify the possible contributing factors to the effects of HH on these afferents.

In most of the experimental models of hemorrhage, a mean systemic arterial pressure (MSAP) of 35–40 mmHg maintained for various durations was used to study the pathophysiology and therapeutic interventions (3). To regulate the extent and duration of HH in this study, we bled the animal to lower the MSAP to ~40 mmHg and then maintained it at a constant level for 20 min before infusing the blood back to the animal for recovery.

METHODS

All protocols were performed in accordance with *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health and were also approved by the University of Kentucky Institutional Animal Care and Use Committee.

Animal preparation. Male Sprague-Dawley rats (n = 56; body weight, 396 ± 6 g) were initially anesthetized with α-chloralose (0.1 mg/g ip) and urethane (0.5 mg/g ip) (Sigma-Aldrich, St. Louis, MO), and supplemental doses (one-tenth of the initial doses) of the same anesthetics were injected intravenously (iv) to maintain abolition of pain reflexes induced by paw pinch. A tracheal tube was inserted after a tracheotomy. The left jugular vein was cannulated by a PE-50 tubing for administering anesthetics and chemical agents. Both femoral arteries were cannulated: one was connected to a pressure transducer (Statham P23AC, Hato Rey, Puerto Rico) for measurements of systemic arterial pressure (SAP) and heart rate (HR), and the other one was connected to a blood reservoir for the HH challenge. Body temperature was maintained at ~36°C by a heating pad connected to a warm-water circulator (Baxter K-MOD 100, Deerfield, IL). At the

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end of experiment, the animals were euthanized by intravenous injections of KCl.

Hemorrhagic hypotension. During the HH challenge, arterial blood was bled from the femoral arterial catheter into a reservoir containing 0.1 ml of 1,000 U/ml heparin (APP Pharmaceuticals, Schaumburg, IL) to avoid blood clotting. The height of the reservoir was lowered until the MSAP reached ~40 mmHg and maintained for 20 min. After the HH challenge, the blood in the reservoir was returned in its entirety to the rat via the venous catheter by gravity initially and completed by infusion with a sterile syringe. The MSAP changed progressively and slowly after the change in reservoir height. No measurement was made until after the MSAP reached a steady-state level, which was considered as the onset (0 min) of the HH challenge or the recovery period.

Recording of pulmonary C-fiber activity. Afferent activities of single-unit vagal pulmonary C-fibers were recorded, as described in details previously (13). Briefly, the lung was artificially ventilated with room air by a constant-volume respirator (Ugo Basile model 7025, Comerio, Italy), and a midline thoracotomy was performed; the expiratory outlet of the respirator was placed under 3 cmH_2O pressure to maintain a near-normal functional residual capacity (FRC). Tidal volume (V_T) and frequency (f) were set at 8 ml/kg and 60 breaths/min, respectively. The caudal end of the cut right vagus nerve was placed on a small dissecting platform and immersed in mineral oil. A thin filament was teased away from the desheathed nerve trunk and further split until the afferent activity arising from a single unit was electrically isolated. The nerve trunk was ligated just above the diaphragm to eliminate afferent signals arising from lower visceral organs.

Pulmonary C-fiber activity was searched initially by their mild responses to lung inflation that was performed by delivery of three or four consecutive V_T via the respirator after occluding the expiratory flow at FRC and by an immediate (delay $<$ 1 s) response to bolus injection of capsaicin (Cap; 0.50–1.00 μg/kg) into the right atrium (13). The general locations of C-fiber terminals were identified by their responses to gentle pressing of the lungs with a blunt-end glass rod at the end of the experiment, which could not, however, exclude the possibility that some of these endings might innervate the small intrapulmonary airways receiving blood supply from pulmonary circulation (13). Action potential (AP), tracheal pressure (P_{tr}), and SAP were recorded on a thermal writer (Gould TW11, Cleveland, OH) and analyzed by a data acquisition system (Biocybernetics TS-100, Taipei, Taiwan) at a sampling rate of 3,000 Hz in this study.

Measurement of oxidative stress. To determine whether oxidative stress was generated by HH, protein carbonyls, the products of oxidized protein side chain (especially of Pro, Arg, Lys, and Thr), were chosen as the markers of oxidative modification of proteins. These moieties are chemically stable for both their detection and storage (6). The concentration of protein carbonyl in the plasma was measured by a commercial colormetric kit (Cayman Chemicals, Ann Arbor, MI). Briefly, venous blood samples (1.0 ml) were drawn from anesthetized rats (artificially ventilated) by syringes coated with heparin (Innovative Med Tech, Leawood, KS) during control, at the end of HH, and at the end of recovery. The blood samples were centrifuged at 1,000 *g* for 10 min at 4°C to collect the plasma samples. Each sample was loaded in two 2-ml vials (200 μl each) and mixed with 800 μl of 2,4-dinitrophenylhydrazine (as sample) or 800 μl of 2.5 M HCl (as control). After 1 h of incubation (brief vortex every 15 min during incubation) in the dark, both vials were placed on ice and washed by 20% trichloroacetic acid (TCA), 10% TCA, and then three times of ethanol-ethyl acetate (1:1), followed by a centrifugation (10,000 *g* for 10 min at 4°C). After the final wash, protein pellets were resuspended by 500 μl of guanidine hydrochloride, and vials were centrifuged again to remove leftover debris. A spectrophotometer was then used to measure the absorbance of each vial at 385 nm.

Experimental design and protocol. Four series of experiments were carried out. *Study 1* was carried out to determine the effects of the HH challenge on MSAP, mean pulmonary arterial pressure (MPAP), and HR and to measure the changes in blood gases in the systemic and pulmonary blood. To measure the MPAP, a catheter was inserted via a jugular venous catheter into the pulmonary artery in 4 rats surgically prepared in the same manner as in *study 2*; the tip position of the catheter was verified by monitoring the pressure signal as it was advanced from right ventricle into pulmonary artery. In a separate series, blood samples (~0.1 ml) were drawn from both the aorta (via a femoral arterial catheter) and right ventricle (via a jugular venous catheter) before and at 20 min after both onset and termination of the HH challenge and placed into test cartridges (Abaxis $CG4^+$, Union City, CA) for blood gas analysis (Abaxis i-STAT 1). In *study 2*, we investigated the effects of HH on pulmonary C-fiber responses to chemical and mechanical stimulations. Chemical stimulations were applied by intravenous bolus injections of several known chemical stimulants of rat vagal pulmonary C-fibers: Cap (0.25–1.00 μg/kg), a

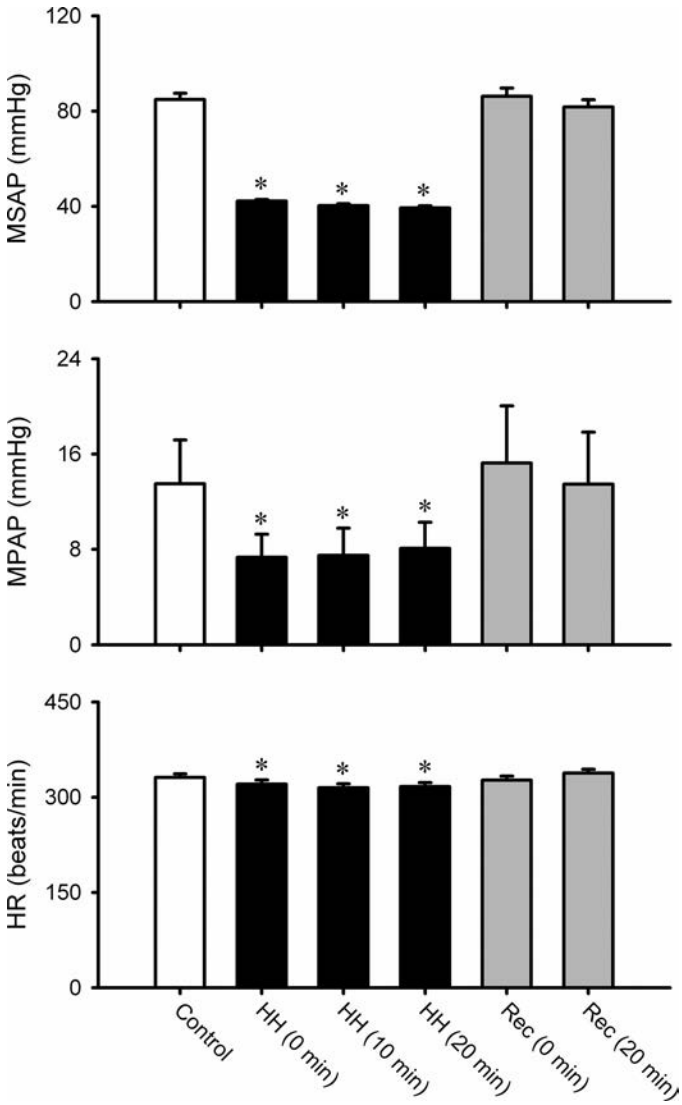

Fig. 1. Effects of the hemorrhagic hypotension (HH) challenge on blood pressure and heart rate (HR, $n = 46$) in anesthetized, open-chest and artificially ventilated rats. MSAP, mean systemic arterial pressure ($n = 46$); MPAP, mean pulmonary arterial pressure ($n = 4$). Control, before the HH challenge; HH (0, 10, or 20 min), responses obtained after the MSAP reached a steady state of HH (~40 mmHg) for 0, 10, or 20 min; Rec (0 or 20 min), responses obtained after the MSAP was returned to the control level for 0 or 20 min during recovery. Note that MPAP was only measured in 4 rats. *Significantly different from control ($P < 0.05$). Data are means ± SE.

selective activator of the transient receptor potential vanilloid type 1 receptor; adenosine (Ado; 0.1–0.3 mg/kg), an activator of the A1 adenosine receptor (14); lactic acid (LA; 5.0–10.0 mg/kg), an activator of acid-sensing ion channel (15); and phenylbiguanide (PBG; 1.0–8.0 μg/kg), an activator of the 5-hydroxytryptamine type 3 receptor (5). The mechanical stimulation was applied by hyperinflation of the lung (HI) with a constant P_{tr} of 30 cmH_2O (lung volume reaching ~3 × V_T above FRC) and maintained for 10 s. *Study 3* was carried out to determine whether oxidative stress was induced by the HH challenge. Venous blood samples were collected and their plasma concentrations of protein carbonyl were measured as described above. In *study 4*, we tested whether the HH-induced pulmonary C-fiber hypersensitivity was caused by production of lactic acid resulting from tissue hypoxia. To alleviate systemic acidosis, solution of sodium bicarbonate ($NaHCO_3$) was infused intravenously at a rate of 86 μmol·kg^{-1}·min (range: 70–200) in a total volume of 3 ml for 30 min; the infusion was initiated as soon as the blood reservoir was lowered. To determine whether the HH-induced C-fiber hypersensitivity could be attenuated by increasing the oxygen intake in the lung, we continuously delivered 100% oxygen to the lung via the ventilator before, during, and after the HH challenge in another group of rats.

Preparation of chemical solutions. The stock solution of Cap (250 μg/ml) was prepared in 10% Tween 80, 10% ethanol, and 80% saline and that of PBG (1 mg/ml), LA (300 mg/ml), and $NaHCO_3$ (10 mM/ml) were prepared in isotonic saline (0.9% NaCl). These stock solutions were stored at −20°C and prepared daily at the desired concentrations for injection by dilution with isotonic saline based on the animal's body weight. Ado was dissolved in sterile water and prepared daily before the experiment. All these chemicals were purchased from Sigma-Aldrich.

Data analysis. The baseline fiber activity (FA) was averaged over a 20-s interval. ΔFA was calculated as the difference between peak FA (averaged over 2-s and 10-s intervals for chemical stimulations and lung inflation, respectively) and the averaged baseline FA in each fiber.

Data were analyzed statistically with either one-way or two-way repeated measures ANOVA, and pair-wise comparisons were made with a post hoc analysis (Fisher's least significant difference). A P value of <0.05 was considered significant. Data are reported as means ± SE.

RESULTS

After the reservoir was lowered to initiate bleeding, the MSAP declined progressively and slowly; the time required to reach a new steady-state level (~40 mmHg) varied among animals and its average was 7.8 ± 0.5 min. To normalize the time of data calculation between animals, the time after the MSAP reached steady state was considered as the onset (0 min) of the HH challenge. During the recovery when the blood in the reservoir was returned in its entirety to the rat, the MSAP also increased slowly and reached a steady state after 8.2 ± 0.3 min, which was considered as the beginning (0 min) of the recovery period.

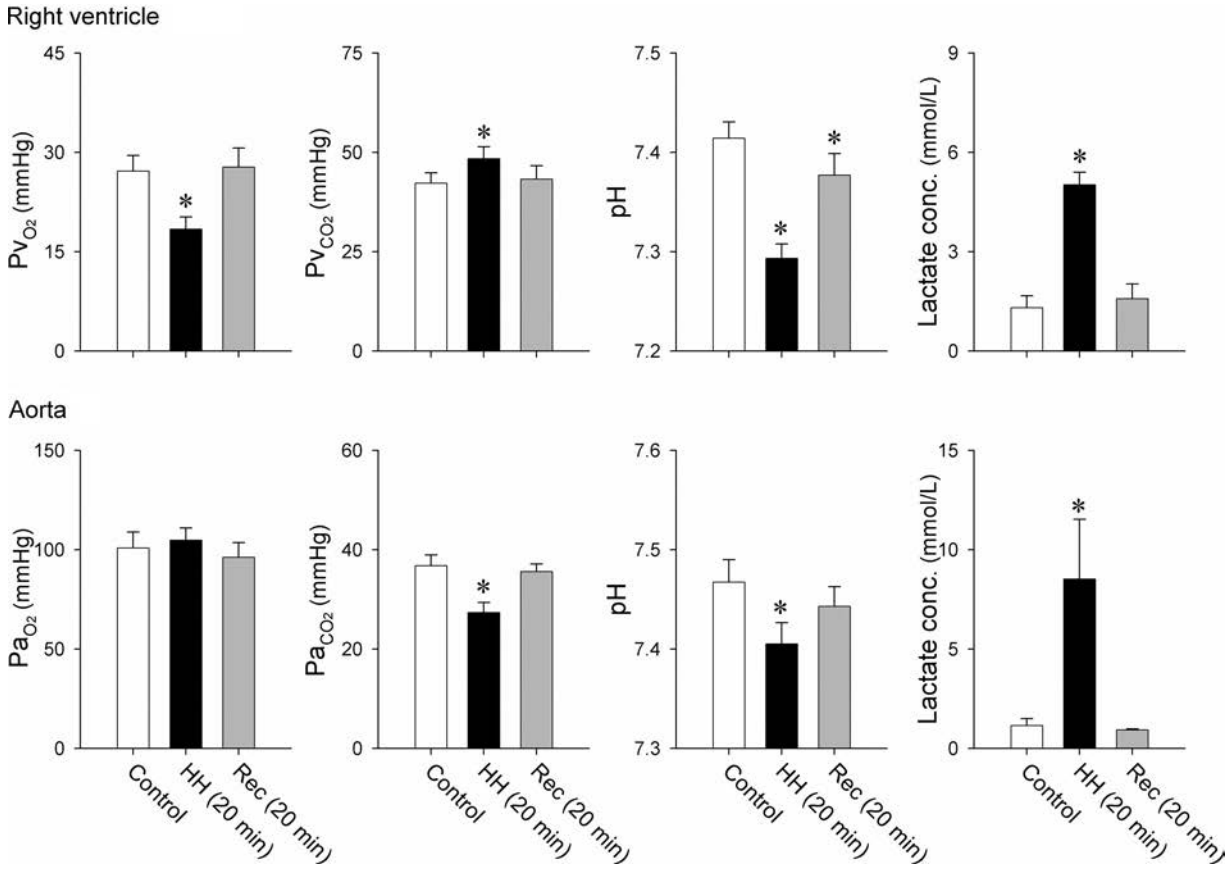

Fig. 2. Changes in blood gases in the systemic and pulmonary blood induced by the HH challenge in anesthetized, open-chest and artificially ventilated rats. *Top*: partial pressures of O_2 (Pv_{O2}) and CO_2 (Pv_{CO2}), pH, and lactate concentration in the mixed venous blood drawn from a jugular venous catheter placed in the right ventricle. *Bottom*: partial pressures of O_2 (Pa_{O2}) and CO_2 (Pa_{CO2}), pH, and lactate concentration in the systemic arterial blood drawn from a femoral arterial catheter with its tip placed in the aorta. Control, before the HH challenge; HH (20 min), after the MSAP reached a steady state of HH (~40 mmHg) for 20 min; Rec (20 min), after the MSAP was returned to the control level for 20 min during recovery. *Significantly different from the corresponding control ($P < 0.05$; $n = 5$ in each group). Data are means ± SE.

Study 1: systemic effects of HH challenge. At the onset of HH, when MSAP was dropped from 84.9 ± 2.6 to 42.3 ± 0.6 mmHg ($P < 0.05$, $n = 46$; Fig. 1), the MPAP decreased from 13.5 ± 3.7 to 7.3 ± 2.0 mmHg ($P < 0.05$, $n = 4$), and HR decreased slightly but significantly from 331 ± 6 to 320 ± 7 beats/min ($P < 0.05$, $n = 46$); data of MSAP and HR also included those collected in *studies 2* and *4*, in which the same surgical preparations as in this study series were performed. When HH was maintained for 20 min, the MSAP, MPAP, and HR remained relatively stable at 39.3 ± 0.9 mmHg, 8.1 ± 2.2 mmHg, and 316 ± 6 beats/min, respectively. MSAP, MPAP, and HR all returned to near the control levels during the recovery period (Fig. 1).

Blood gases in arterial and venous blood were measured in 5 rats. After HH was maintained for 20 min, blood samples drawn from the right ventricle (mixed venous blood) showed a decrease of the partial pressure of O_2 (Pv_{O2}) by ~32.7% ($P < 0.05$, $n = 5$), an increase of Pv_{CO2} by ~14.5% ($P < 0.05$, $n = 5$), and a reduction of pH (an increase of H^+ concentration by 32.16%; $P < 0.05$, $n = 5$), accompanied by a large increase of lactate concentration by ~353.8% ($P < 0.05$, $n = 5$; Fig. 2). All these changes except venous pH showed full recovery 20 min after the termination of HH (Fig. 2, *top*). In contrast, at the same time points, the systemic arterial blood showed no change in Pa_{O2} from the baseline ($P > 0.05$, $n = 5$), a decrease of Pa_{CO2} by ~24.9% ($P < 0.05$, $n = 5$), a reduction of pH (an increase of H^+ concentration by 15.55%; $P < 0.05$, $n = 5$), and a pronounced increase in the lactate concentration by ~882.1% ($P < 0.05$, $n = 5$). All these changes showed full recovery 20 min after the termination of HH (Fig. 2, *bottom*).

Study 2: effect of HH on pulmonary C-fiber sensitivity. Pulmonary C-fibers were generally quiescent during the baseline at control (Figs. 3 and 4). The baseline FA of pulmonary C-fibers increased progressively from 0 ± 0 imp/s at the

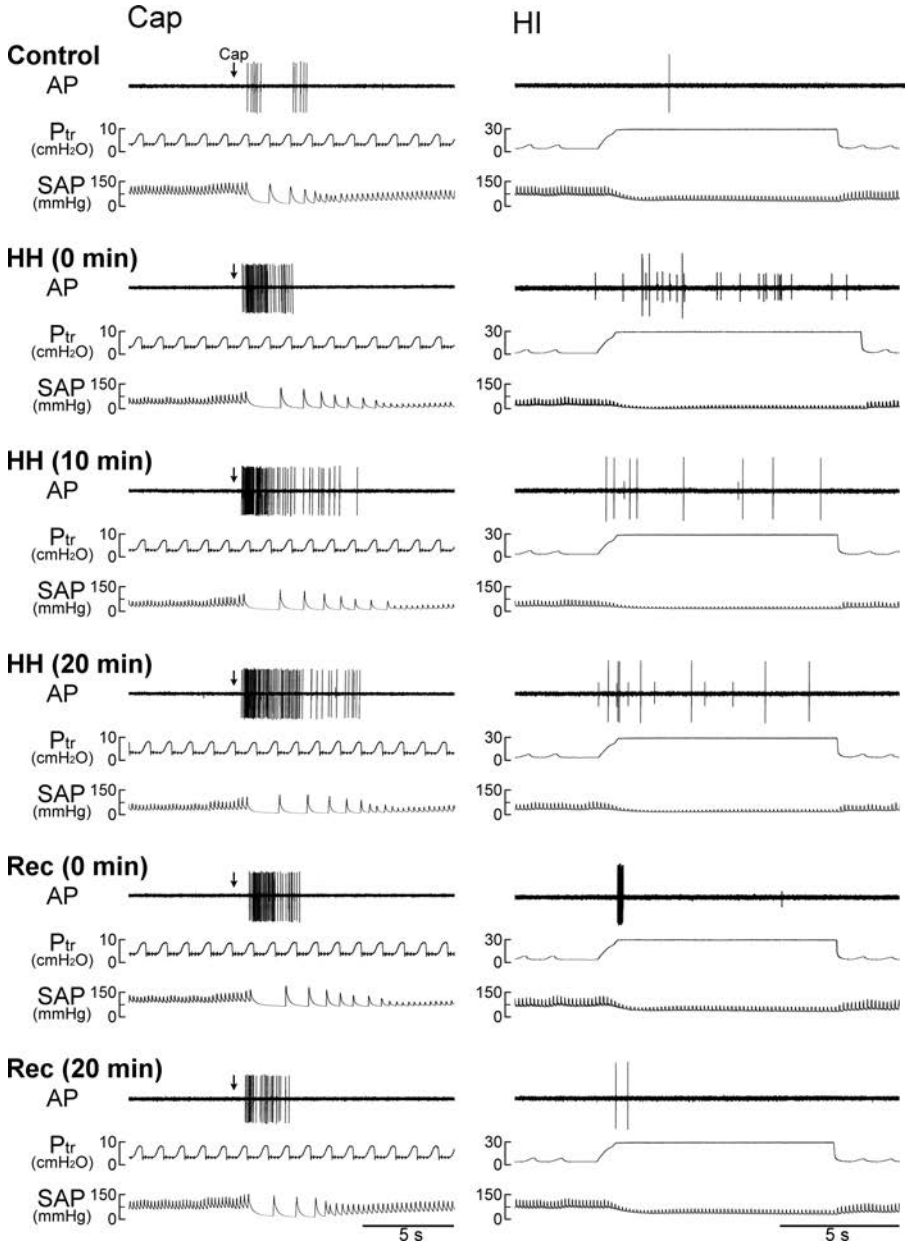

Fig. 3. Experimental records illustrating the effect of HH on the responses of two pulmonary C-fibers to capsaicin (Cap; 1.00 μg/kg) and lung hyperinflation (HI; 30 cmH_2O maintained for 10 s) in anesthetized, open-chest and artificially ventilated rats. Cap solution (0.15 ml volume) was delivered first into the catheter (dead space volume 0.2 ml) and then flushed (at arrow) into the circulation by a bolus of 0.3 ml saline. Control, before HH; HH (0, 10, or 20 min), responses obtained after the MSAP reached a steady state of HH for 0, 10, or 20 min; Rec (0 or 20 min), responses obtained after the MSAP was returned to the control level for 0 or 20 min during recovery. AP, action potential; P_{tr}, tracheal pressure; SAP, systemic arterial pressure. Notice that in the right, there was a second C-fiber with smaller amplitude of AP; it responded to Cap injection (data not shown) and was activated vigorously by HI during the HH challenge. Receptor locations: upper lobe (*left*) and middle lobe (*right*). Body weights: 350 g (*left*) and 390 g (*right*).

beginning of the HH challenge to a peak of 0.064 ± 0.039 imp/s at the end of 20 min HH ($P < 0.05$, $n = 11$; Fig. 5, *left*). During the recovery period, baseline FA gradually decreased and returned to control after the termination of HH for 20 min.

At control (before HH), the bolus injection (iv) of a low dose of Cap triggered a short and mild burst of discharge of pulmonary C-fibers (e.g., Fig. 3). After MSAP reached the steady state of HH, ΔFA triggered by the same dose of Cap increased from 2.18 ± 0.56 imp/s at control to 10.82 ± 1.72 imp/s at 0 min of HH ($P < 0.05$, $n = 11$); the increased ΔFA continued at 10 min and reached the peak at 20 min of the HH challenge (15.02 ± 1.94 imp/s, $P < 0.05$, $n = 11$; Fig. 5, *middle*). This pulmonary C-fiber hypersensitivity to Cap was accompanied by intensified bradycardia (Figs. 3 and 4). The increased sensitivity remained significantly higher than control immediately after the MSAP returned to control (8.26 ± 1.98 imp/s, $P < 0.05$, $n = 11$; Fig. 5, *middle*) and slowly returned to near the control level 20 min later (Fig. 4, *top*, and Fig. 5, *middle*).

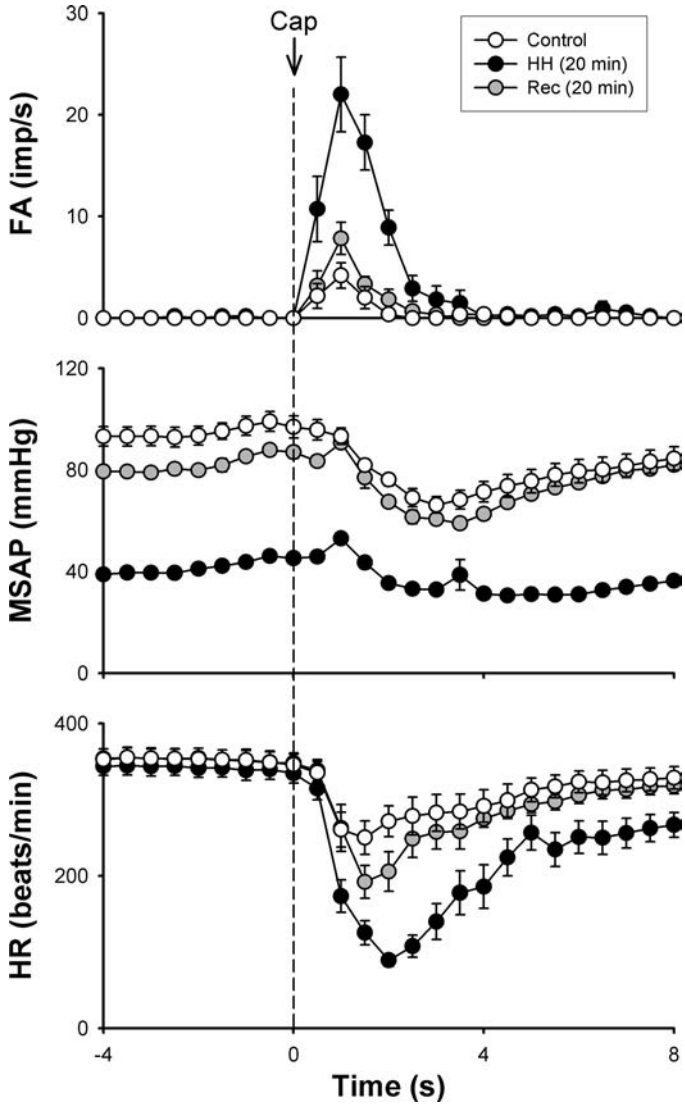

Fig. 4. Effects of HH on the responses of pulmonary C-fibers (FA) to Cap (0.25–1.00 μg/kg; *top*), MSAP (*middle*), and HR (*bottom*) in anesthetized, open-chest and artificially ventilated rats. Control (open circles), before HH; HH (20 min), responses obtained after the MSAP reached a steady state of HH for 20 min (closed circles); Rec (20 min), responses obtained after the MSAP was returned to the control level for 20 min during recovery (shaded circles). Cap was injected as an intravenous bolus at 0 s. Data are means ± SE; $n = 11$.

A pronounced potentiating effect of the HH challenge was also found consistently in pulmonary C-fiber responses to all other chemical stimulants of pulmonary C-fibers tested in this study: Ado ($n = 9$), LA ($n = 8$), and PBG ($n = 8$) (Fig. 6). It should be noted that there was a difference in the magnitude and pattern of the HH-induced potentiation between the C-fiber responses to these chemical stimulants; for example, at 0 min of HH, the responses to Ado, LA, and PBG were elevated to 123%, 177%, and 688% of their control responses, respectively (Fig. 6). The ΔFA evoked by bolus injection of vehicle (isotonic saline) was 0.17 ± 0.11 imp/s at control and 0.08 ± 0.08 imp/s after the HH challenge was maintained for 20 min ($P > 0.05$, $n = 6$).

Pulmonary C-fibers exhibited low sensitivity to HI at control (before HH). However, the FA response to HI (P_{tr} = 30 cmH_2O; 10-s duration) increased progressively during the HH challenge: the HI-evoked ΔFA increased from 0.35 ± 0.12 imp/s at control to 0.98 ± 0.32 imp/s ($P < 0.05$, $n = 8$) at 10 min, and 1.08 ± 0.33 imp/s ($P < 0.05$, $n = 8$) at 20 min of HH. This elevated sensitivity to HI persisted even after the MSAP returned to control after HH and gradually returned to the control level 20 min later (Fig. 5, *right*).

Study 3: effect of HH on oxidative stress. The plasma concentrations of protein carbonyl were 26.55 ± 0.89, 21.13 ± 0.72, and 23.26 ± 0.90 nmol/ml for control, after 20 min of HH, and after 20 min of recovery, respectively ($P < 0.05$, $n = 6$). However, total protein also showed a similar trend of decrease: 54.54 ± 2.79, 43.00 ± 1.76, and 47.74 ± 0.76 mg/ml at the corresponding time points ($P < 0.05$, $n = 6$). Thus the plasma protein carbonyl content per unit of total protein did not change during or after the HH challenge: 0.49 ± 0.03, 0.49 ± 0.02, and 0.49 ± 0.02 nmol/mg for control, at 20 min after the onset of HH, and at 20 min after the termination of HH, respectively ($P > 0.05$, $n = 6$).

Study 4: possible influences of systemic acidosis and tissue hypoxia induced by HH. Infusion of $NaHCO_3$ (86 μmol·kg^{-1}·min iv) for 30 min during the HH challenge significantly attenuated the systemic acidosis, measured by the reduction in venous blood pH at the end of the HH challenge (Fig. 7, *middle*), but it did not change the HH-induced lactate production (Fig. 7, *right*). Accompanying the change in venous blood pH, infusion of $NaHCO_3$ significantly attenuated the HH-induced C-fiber hypersensitivity to Cap at the end of the HH challenge ($P < 0.05$, $n = 8$; Fig. 7, *left*).

Ventilation with 100% O_2 did not significantly change either P_{O_2} or lactate concentration in the mixed venous blood at the end of the HH challenge ($P > 0.05$, $n = 5$; Fig. 8, *middle* and *right*), and it also failed to alter the C-fibers hypersensitivity to Cap induced by the HH challenge ($P > 0.05$, $n = 7$; Fig. 8, *left*).

DISCUSSION

This study clearly demonstrated that vagal pulmonary C-fiber sensitivity was markedly elevated during HH. The C-fiber hypersensitivity developed at the onset of the HH challenge increased progressively during HH and persisted even after the arterial blood pressure returned to the control level during recovery. The increased sensitivity was found in their responses to both Cap injection and HI; the latter is of particular

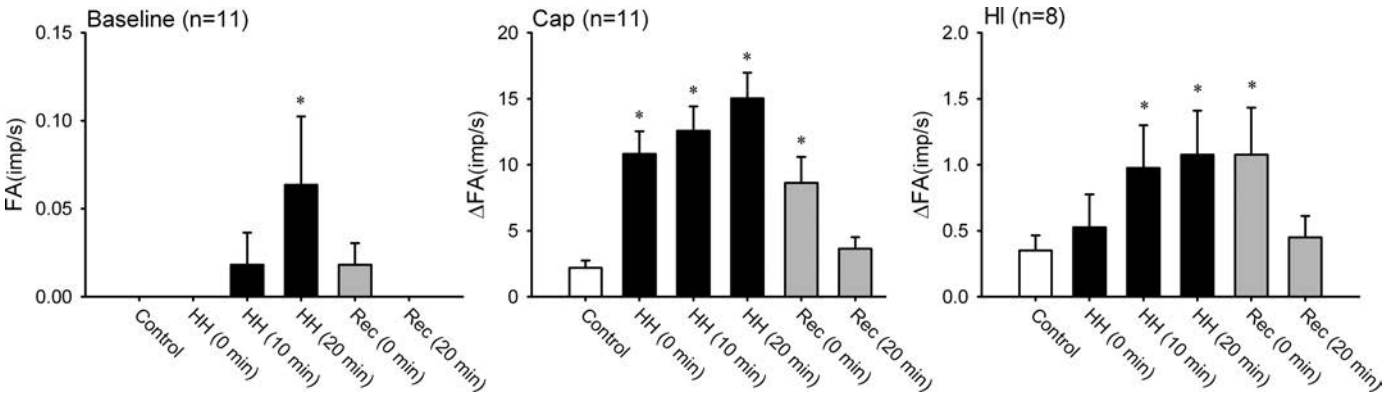

Fig. 5. Effects of HH on the pulmonary C-fiber baseline activity and on the pulmonary C-fiber responses to Cap (0.25–1.00 μg/kg) and HI (tracheal pressure maintained at 30 cmH_2O for 10 s) in anesthetized, open-chest and artificially ventilated rats. FA, averaged baseline fiber activity over 20-s interval. ΔFA, the difference between peak fiber activity (averaged over 2-s and 10-s intervals for Cap and HI, respectively) and averaged baseline FA (20-s interval) in each fiber. Control, before the HH challenge; HH (0, 10, or 20 min), responses obtained after the MSAP reached a steady state of HH (~40 mmHg) for 0, 10, or 20 min; Rec (0 or 20 min), responses obtained after the MSAP was returned to control for 0 or 20 min during recovery. *Significantly different from control baseline or response ($P < 0.05$). Data are means ± SE.

interest because lung expansion is a natural physiological action, and pulmonary C-fibers normally have low sensitivity to HI (13, 19). In addition, the increased chemical sensitivity induced by HH in these afferents was not limited to Cap but was also evident in their responses to other chemical stimulants of C-fibers tested in this study: Ado, PBG, and LA; each of them represents a selective activator of a specific type of receptor expressed in the pulmonary C-fiber sensory endings. The hypersensitivity of pulmonary C-fiber afferents to these chemical stimulants was accompanied by intensified bradycardia (e.g., Figs. 3 and 4), probably resulting from the reflexes elicited by stimulation of C-fibers in the contralateral lung. Although the mechanism(s) underlying this effect generated by HH could not be determined in this study, several potential contributing factors should be considered.

It has been shown that acute severe hypoxia can enhance the C-fiber sensitivity to Cap (30). The HH challenge was expected to induce systemic tissue hypoxia, which was confirmed by a substantial decrease in P_{O_2} in the mixed venous blood, despite no change in the arterial P_{O_2} (Fig. 2); the tissue hypoxia presumably resulted from a reduction of the blood flow and oxygen delivery to peripheral tissues. The C-fiber endings are located in the lung parenchyma that receives blood supply from pulmonary circulation (5) and, therefore, responsive to the changes in chemical compositions of the mixed venous blood (from pulmonary artery) and not that in the systemic arterial blood. Ventilation with 100% O_2 did not significantly attenuate the tissue hypoxia and lactic acid production (Fig. 8). The lack of effect is not unexpected because the increase in arterial O_2 content by oxygen ventilation is limited to the minute increase in dissolved O_2, which cannot offset the deficit in O_2 delivery to the tissue resulting from the reduced blood supply during HH. The fact that ventilation with O_2 did not significantly alter the C-fiber hypersensitivity induced by the HH challenge (Fig. 8, *left*) further suggests a possible contribution of tissue hypoxia to the increased C-fiber sensitivity during HH. Indeed, both lactic acid and adenosine are produced by anaerobic metabolism during tissue hypoxia, and both have been shown to exert a potent stimulatory and sensitizing effects on rat pulmonary C-fiber afferents in our previous studies (12, 14, 15). The fact that a distinct increase in lactate concentration and P_{O_2} in the mixed venous blood (Fig. 2) clearly indicate that HH induces tissue hypoxia in this study. Furthermore, HH is known to stimulate sympathetic activity and increase the levels

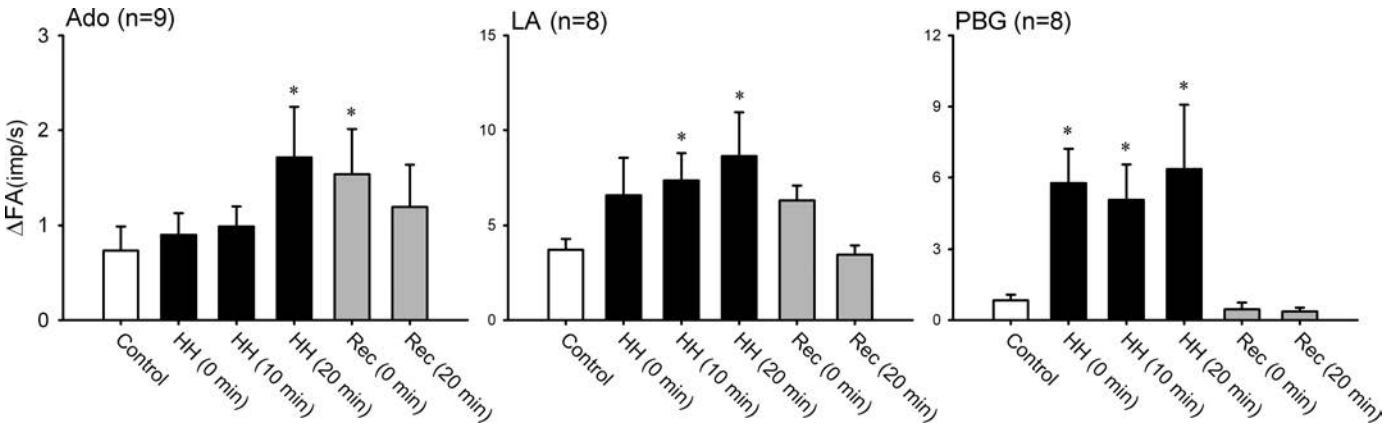

Fig. 6. Effects of HH on the pulmonary C-fiber responses to adenosine (Ado; 0.1–0.3 mg/kg), lactic acid (LA; 5.0–10.0 mg/kg), and phenylbiguanide (PBG; 1.0–8.0 μg/kg) in anesthetized, open-chest and artificially ventilated rats. For the explanations of calculation of ΔFA and time points of measurements, see legend of Fig. 5. *Significantly different from the corresponding control response ($P < 0.05$). Data are means ± SE.

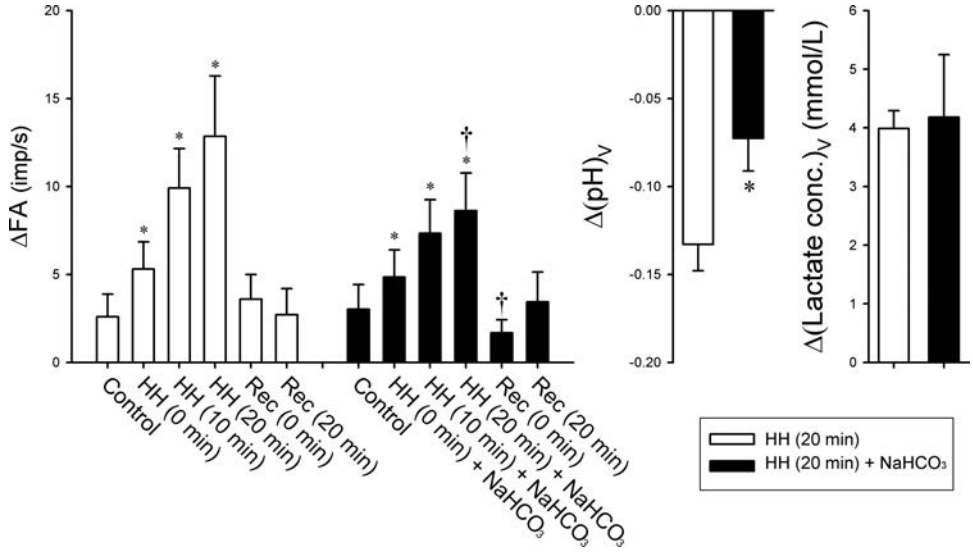

Fig. 7. Effects of HH on pulmonary C-fiber responses to Cap (0.25–1.00 μg/kg, *left*) and changes in mixed venous blood pH (*middle*) and lactate concentration (*right*). Intravenous infusion of sodium bicarbonate solution ($NaHCO_3$; 86 μmol·kg^{-1}·min for 30 min) was initiated as soon as the blood reservoir was lowered to induce HH. Closed bars, responses obtained during the $NaHCO_3$ infusion. Open bars, responses obtained in the same rats without infusion of $NaHCO_3$. For the explanations of calculation of ΔFA and time points of measurements, see legend of Fig. 5. *Significantly different ($P < 0.05$) from the corresponding control response ($n = 8$ in *left*; $n = 6$ in *middle* and *right*); †Significantly different ($P < 0.05$) from the corresponding data point in the non-$NaHCO_3$ group. Data are means ± SE.

of circulatory catecholamines, and epinephrine has been shown to enhance the sensitivity of pulmonary C-fibers to Cap and HI via an activation of β_3-adrenoceptor (11).

The sensitivity of pulmonary C-fiber afferents can be also elevated by an increase in CO_2 in the alveolar air and pulmonary blood, and the action can be prevented by a pretreatment with $NaHCO_3$ to reverse the acidosis, indicating the involvement of hydrogen ion (10). In this study, both acidosis and hypercapnia were found in the pulmonary arterial blood (systemic mixed venous blood) drawn from the right ventricle during HH, and these changes were reversible after MSAP returned to control (Fig. 2, *top*). In addition, the blood lactate concentration was also markedly elevated during HH (Fig. 2, *top*), suggesting an excessive production of lactic acid probably caused by tissue hypoxia and anaerobic metabolism. Interestingly, when infusion of $NaHCO_3$ alleviated the HH-induced systemic acidosis, it also significantly attenuated the C-fiber hypersensitivity at the end of the HH challenge (Fig. 7, *left*). These results seem to suggest a possible involvement of hydrogen ion in the augmented C-fiber sensitivity induced by the HH challenge.

MPAP was substantially reduced during HH (Fig. 1). Although the change in pulmonary blood flow caused by the hemorrhage was not measured in this study, presumably it was also reduced during HH due to the loss of total blood volume. If so, for the same injected chemical solution, its concentration in the pulmonary circulation may have been higher during HH than control, which could have contributed, in part, to the enhanced sensitivity. However, we do not believe this to be a major contributing factor for the following reasons. First, the hypersensitivity of pulmonary C-fiber induced by HH was not limited to intravenous injections of chemical stimulants; a similar potentiation was also observed in their response to HI during HH (Figs. 3 and 5). Second, the C-fiber responses to chemical challenges showed a trend to increase progressively during the 20 min of HH. In fact, immediately after the MSAP was lowered to 40 mmHg (at the beginning of the HH challenge), there was no significant increase in C-fiber responses to some of the chemical stimulants (e.g., Ado, LA; Fig. 6). Third, the hypersensitivity of pulmonary C-fibers persisted even after the MSAP had already returned completely to the control level during recovery. Finally, in our pilot study of the effect of HH

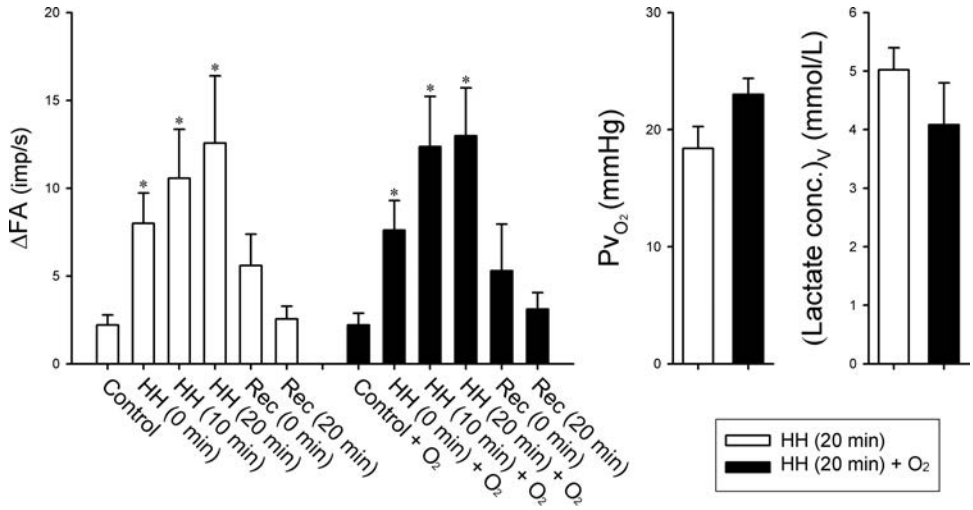

Fig. 8. Effects of HH on pulmonary C-fiber responses to Cap (0.25–1.00 μg/kg, *left*), changes in mixed venous blood P_{O_2} (*middle*), and lactate concentration (*right*) with and without 100% O_2 ventilation. Open bars, responses obtained when rats were ventilated with room air. Closed bars, responses obtained in the same rats during O_2 ventilation. For the explanations of calculation of ΔFA and time points of measurements, see legend of Fig. 5. *Significantly different ($P < 0.05$) from the corresponding control response ($n = 7$ in *left*; $n = 5$ in *middle* and *right*). Data are means ± SE.

on pulmonary chemoreflex responses elicited by C-fiber stimulation, the potentiating effect of HH was also clearly observed when Cap was delivered by aerosol inhalation (instead of intravenous injection; data not shown).

It is well documented that severe hemorrhage can induce systemic oxidative stress and trigger local release of ROS resulting from tissue ischemia-reperfusion (26). Some of these ROS, such as hydrogen peroxide and hydroxyl radicals, can activate pulmonary C-fibers (24, 25). To test a possible involvement of ROS in the HH-induced C-fiber hypersensitivity, we measured the plasma protein carbonyl content, one of the most widely used markers of oxidative stress (4, 6), and compared between before, during, and after the HH challenge. However, we did not detect any increase in the plasma level of protein carbonyl during either HH or the recovery phase. Although we cannot completely rule out a possible involvement of a lower level of ROS undetected by protein carbonyl in our study, it is unlikely that oxidative stress plays a major part in the elevated C-fiber sensitivity in this study.

Another mechanism possibly involved in the HH-induced C-fiber hypersensitivity is the systemic inflammation caused by hemorrhage. It is well documented that acute hemorrhage causes a surging production of pro-inflammatory cytokines from macrophages and other cells of innate immune system (3). For example, pronounced increases in the plasma levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, and IL-10 have been reported in the studies when hemorrhage of similar intensity and duration of hypotension as that in our study was induced (1). Some of these cytokines such as TNF-α and IL-1β are known to exert potent stimulatory and/or sensitizing effects on vagal pulmonary C-fiber afferents (16, 22, 31).

These C-fiber afferents are known to play an important role in the pulmonary defense function. Activation of these afferents by inhaled irritants (e.g., cigarette smoke, sulfur dioxide, ozone, etc.) or endogenous chemical mediators (e.g., hydrogen ion, adenosine 5′-triphosphate, bradykinin, eosinophil granule-derived cationic proteins, etc.) can elicit powerful protective reflex responses, such as cough, mucus secretion, bronchoconstriction, dyspneic sensation, and J reflex (5, 21). Furthermore, these sensory nerves have been recognized to play an important role in the neural-immune interaction in response to various airway assaults (e.g., allergens, irritant chemicals) (21, 28); for example, neuropeptides (e.g., substance P) released from pulmonary C-fiber endings upon activation can interact with a number of immune cells (e.g., macrophage, mast cell) and modulate their activity and cytokine release (21, 28). Hence, their sensitivity is not only important in protecting the lung and body against potential health hazardous actions caused by air-borne chemical irritants, but also in regulating airway function under various pathophysiological conditions (5, 21). Our results obtained in this study would suggest that these protective functions of the lung are heightened during hemorrhage when the body is more susceptible to other hazardous insults and pathophysiological stresses.

Perspectives and Significance

This study demonstrated that HH induced an elevated sensitivity of pulmonary C-fiber afferents to various chemical stimuli and lung inflation, which persisted even when the MSAP had returned to the control level after the termination of HH. We believe that the hypersensitivity was probably generated by the endogenous release of inflammatory mediators and chemical substances including hydrogen ion that are produced by tissue ischemia/hypoxia during HH. The elevated C-fiber sensitivity may be important in enhancing the pulmonary defense function during this pathophysiological condition. In addition, it may also play a part in the vagal anti-inflammatory mechanism (28, 29) that inhibits the synthesis and release of pro-inflammatory cytokines, thereby preventing further tissue damage caused by hemorrhage and tissue ischemia.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: R.-L.L., Y.-J.L., F.X., and L.-Y.L. conception and design of research; R.-L.L. and Y.-J.L. performed experiments; R.-L.L., Y.-J.L., and L.-Y.L. analyzed data; R.-L.L., Y.-J.L., F.X., and L.-Y.L. interpreted results of experiments; R.-L.L., Y.-J.L., and L.-Y.L. prepared figures; R.-L.L., Y.-J.L., F.X., and L.-Y.L. drafted manuscript; R.-L.L., F.X., and L.-Y.L. edited and revised manuscript; R.-L.L., Y.-J.L., F.X., and L.-Y.L. approved final version of manuscript.

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Short communication

Airway extravasation induced by increasing airway temperature in ovalbumin-sensitized rats

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ABSTRACT

This study was carried out to determine whether hyperventilation of humidified warm air (HWA) induced airway extravasation in ovalbumin (Ova)-sensitized rats. Our results showed: (1) After isocapnic hyperventilation with HWA for 2 min, tracheal temperature (T_{tr}) was increased to 40.3 °C, and the Evans blue contents in major airways and lung tissue were elevated to 651% and 707%, respectively, of that after hyperventilation with humidified room air in Ova-sensitized rats; this striking effect of HWA was absent in control rats. (2) The HWA-induced increase in Evans blue content in sensitized rats was completely prevented by a pretreatment with either L-732138, a selective antagonist of neurokinin type 1 (NK-1) receptor, or formoterol, a selective agonist of β_2 adrenoceptor. This study demonstrated that an increase in airway temperature induced protein extravasation in the major airways and lung tissue of sensitized rats, and an activation of the NK-1 receptor by tachykinins released from bronchopulmonary C-fiber nerve endings was primarily responsible.

1. Introduction

A recent study carried out in our lab reported that hyperventilation (40% of maximal voluntary ventilation for 4 min) of humidified warm air (HWA) triggered cough and bronchoconstriction in patients with mild and stable asthma, and activation of bronchopulmonary sensory nerves by HWA was believed to be responsible (Hayes et al., 2012). A follow-up study further demonstrated that an increase in airway temperature resulting from hyperventilation of HWA evoked a pronounced increase in airway resistance in ovalbumin (Ova)-sensitized Brown–Norway rats, and that endogenous tachykinins were the primary contributing factor (Hsu et al., 2013). Furthermore, the HWA-induced airway response was not prevented by atropine and sustained for >10 min (Hsu et al., 2013), suggesting that the effect was not generated by the reflex-mediated airway smooth muscle contraction in anesthetized rats.

Tachykinins, such as substance P (SP) and neurokinin (NK) A, are pro-inflammatory neuropeptides released from pulmonary C-fiber sensory nerve endings upon intense stimulation. These neuropeptides can trigger a number of neurogenic inflammatory responses in the airways, including protein extravasation, mucosal edema and bronchoconstriction, by activating NK type 1 (NK-1) and type 2 (NK-2) receptors expressed on a number of target cells in the airways, including endothelial cells in capillaries and venules, airway and vascular smooth muscles (Steinhoff et al., 2014). However, whether airway extravasation was evoked by the HWA challenge in Ova-sensitized rats is not known (Hsu et al., 2013).

This study was carried out to answer the following questions: (1) Does hyperventilation of HWA induce airway extravasation, and if so, is the effect augmented in Ova-sensitized rats? (2) Are tachykinins responsible for the HWA-evoked airway extravasation, and if so, what are the relative roles of NK-1 and NK-2 receptors in mediating these responses?

2. Materials and methods

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The study protocol was approved by the University of Kentucky Institutional Animal Care and Use Committee.

2.1. Animal sensitization and preparation

Adult male pathogen-free Brown–Norway rats (267.5 ± 3.3 g) were separated into control and sensitized groups. Sensitized rats received an initial intraperitoneal (ip) injection of a suspension containing 2 mg of Ova (Sigma-Aldrich, St. Louis, MO) in 1 ml of Imject Alum (Pierce Biotechnology, Rockford, IL) as adjuvant. Three

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days later, rats were exposed to 1.25% Ova aerosol for 15 min three times per week (M/W/F) for 3 weeks, following the protocol identical to that described in our previous study (Hsu et al., 2013). Control rats received an ip injection of Imject Alum and inhalation of aerosolized vehicle (isotonic saline). One day after the last Ova or saline exposure, rats were anesthetized with α-chloralose (100 mg/kg; Sigma-Aldrich) and urethane (500 mg/kg; Sigma-Aldrich), placed in a supine position and ventilated mechanically with a respirator (model 683; Harvard, South Natick, MA) via a short tracheal cannula inserted just below the larynx. Respiratory rate (f) was set at 60 breaths/min, and tidal volume (V_T) at 6–7 ml/kg. Body temperature was maintained at 36 °C by a heating blanket. A polyethylene catheter was inserted into the left femoral vein for intravenous (iv) bolus injections of drugs.

2.2. HWA challenge

The method for the HWA challenge was described in details in a previous report (Hsu et al., 2013). Briefly, the outlet of the respirator inspiratory line was connected to an air stone that was immersed in isotonic saline contained in a bottle placed in a heated water bath. HWA was then delivered directly into the lung via the tracheal tube. Humidified room air (HRA) was delivered in the same manner except that the water bath was kept at the room temperature (~23 °C). During both HWA and HRA challenges, minute ventilation was elevated to ~375% of the baseline (by increasing V_T and f to 150% and 250% of their baselines, respectively) for 2 min; ~4.0% of CO_2 was added to the inspired air to maintain the isocapnic condition during hyperventilation. A miniature temperature probe (model IT-18; Physitemp, Clifton, NJ) was inserted into the tracheal tube and positioned near the thoracic inlet to continuously measure the air temperature in the trachea before and during HWA and HRA challenges.

2.3. Measurement of airway extravasation

Evans blue dye (30 mg/kg iv; Sigma-Aldrich) was injected 5 min before the HWA or HRA challenge. Ten min after the HWA or HRA challenge, the thorax was opened. To remove blood and intravascular Evans blue dye, pulmonary circulation and systemic circulation were perfused with isotonic saline via cannulated pulmonary artery (pressure, 30 mmHg; volume, 25 ml) and aorta (pressure, 120 mmHg; volume, 200 ml), respectively. The whole lung including trachea was then removed, separated into major airways (trachea and major bronchi) and lung tissue (lung parenchyma and intrapulmonary airways), and weighted. The intensity of airway extravasation was quantified by measuring the extravasation amount of Evans blue dye (Evans et al., 1988) extracted from tissue in formamide (Sigma-Aldrich) by incubation at 40 °C in water bath for 24 h and measured by light absorbance (SpectraMax, M2; Molecular Devices, Sunnyvale, CA) at 620 nm. The Evans blue content in tissue was determined from a standard curve of the dye in the concentration range of 0.05–20 μg/ml, and expressed as ng Evans blue dye per mg wet tissue (ng/mg).

2.4. Experimental protocols

Four series of experiments were carried out. *Series 1* aimed to determine if airway extravasation was generated by the increase of tracheal temperature (T_{tr}) induced by the HWA challenge; and to compare the responses between control and Ova-sensitized rats. Both control and sensitized rats were divided into two groups ($n = 6$ in each group) for HWA and HRA challenges. *Series 2:* To investigate the role of the endogenous tachykinins, the HWA-induced extravasation responses in Ova-sensitized rats were compared between a control group (pretreated with vehicle) and a group pretreated with a combination of L-732138 (6 mg/kg iv; Tocris, Ellisville, MO), a selective NK-1 antagonist, and SR-48968 (1 mg/kg iv; Sanofi Recherche, Montpellier, France), a selective NK-2 antagonist 20 min earlier. *Series 3*: To determine the relative contributions of NK-1 and NK-2 receptors, the HWA-induced extravasation responses were compared between two groups of Ova-sensitized rats pretreated with L-732138 alone and SR-48968 alone, respectively, 20 min earlier. *Series 4:* To study the role of β_2 adrenoceptors, the HWA-induced extravasation responses were determined in Ova-sensitized rats pretreated with formoterol (10 μg/kg iv; Sigma-Aldrich), a selective β_2 agonist, 60 min earlier.

2.5. Statistical analysis

Data were compared using one-way or two-way analysis of variance (ANOVA), followed by a post hoc Fisher's test. P values of <0.05 were considered significant. Data are reported as means ± SE.

3. Results

A total of 49 rats were used in this study. Despite that the age was matched between the two groups, the average body weight of Ova-sensitized rats (259 ± 3 g; $n = 37$) was significantly lower than that of control rats (293 ± 4 g; $n = 12$, $P < 0.01$). Hyperventilation of HWA elevated T_{tr} from 31.5 ± 0.5 °C to 40.1 ± 0.3 °C ($n = 6$, $P < 0.001$) in control rats and from 31.8 ± 0.2 °C to 40.3 ± 0.1 °C ($n = 6$, $P < 0.001$) in sensitized rats. Hyperventilation with HRA decreased T_{tr} (from 31.3 ± 0.4 °C to 24.4 ± 0.4 °C in control rats; from 31.4 ± 0.3 °C to 24.4 ± 0.2 °C in sensitized rats).

3.1. Series 1

The Evans blue content after HWA challenge was significantly higher than that after HRA in both major airways and lung tissue in Ova-sensitized rats, but not in control rats. In Ova-sensitized rats, Evans blue contents measured in major airways were 143.8 ± 34.4 ng/mg and 22.1 ± 5.3 ng/mg in the two groups receiving HWA and HRA challenges, respectively ($P < 0.01$, $n = 6$; Fig. 1A). In lung tissue, the Evans blue contents were 29.7 ± 5.7 ng/mg and 4.2 ± 0.7 ng/mg in HWA and HRA groups, respectively ($P < 0.01$, $n = 6$; Fig. 1B). In a sharp contrast, in either major airways or lung tissue, the Evans blue contents were not different between the two groups of control rats receiving HWA and HRA challenges ($P > 0.05$, $n = 6$; Fig. 1).

3.2. Series 2

Pretreatment with a combination of L-732138 and SR-48968 completely abolished the increase in Evans blue content in both major airways and lung tissue induced by the HWA challenge in Ova-sensitized rats (Fig. 2). In major airways, the Evans blue contents after HWA were 118.7 ± 11.6 in the control (vehicle) group, and 17.8 ± 1.9 ng/mg in the group pretreated with a combination of L-732138 and SR-48968 ($P < 0.01$, $n = 5$; Fig. 2A); in lung tissue, the Evans blue contents after HWA were 30.9 ± 6.3 and 5.8 ± 0.6 ng/mg in the control (vehicle) and treated (L-732138 + SR-48968) groups, respectively ($P < 0.01$, $n = 5$; Fig. 2B).

3.3. Series 3

Pretreatment with L-732138 alone significantly attenuated the HWA-induced increase in Evans blue content to 20.9 ± 1.0 ng/mg ($P < 0.01$, $n = 5$; Fig. 2A) in major airways, and to 3.8 ± 0.2 ng/mg ($P < 0.01$, $n = 5$; Fig. 2B) in lung tissue in Ova-sensitized rats. In contrast, pretreatment with SR-48968 alone did not significantly change the HWA-induced increase in Evans blue content in major

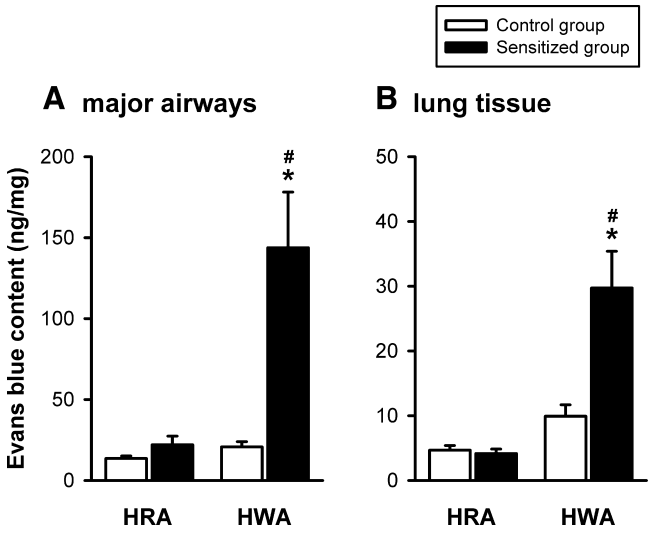

Fig. 1. Comparison of the intensity of airway extravasation in response to hyperventilation with humidified warm air (HWA) and humidified room air (HRA) in control and ovalbumin (Ova)-sensitized rats. Open bars, control groups; filled bars, Ova-sensitized groups. The intensity of protein extravasation was measured in ng of Evans blue content per mg of wet tissue weight (Evans blue content) in (A) major airways (trachea and major bronchi); (B) lung tissue (lung parenchyma and intrapulmonary airways). Data are means ± SE; 6 rats in each group. * Significant difference when corresponding data were compared between control and sensitized groups ($P < 0.05$); # significant difference when corresponding data were compared between HWA and HRA responses ($P < 0.05$).

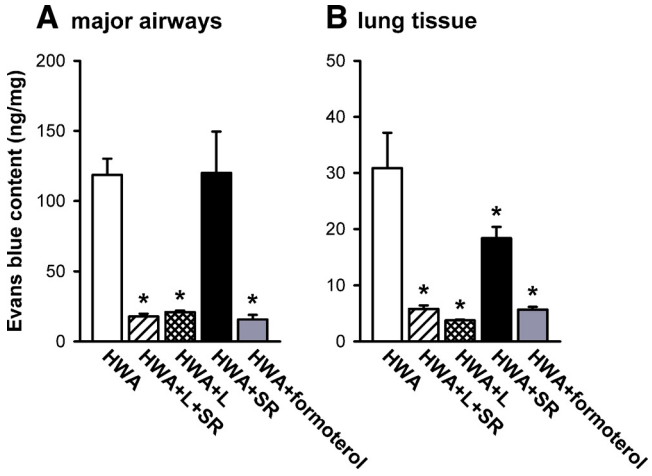

Fig. 2. Role of endogenous tachykinins and formoterol in the HWA-induced airway extravasation in five different groups of Ova-sensitized rats. Open bars, responses to HWA challenge in the control (pretreated with vehicle) group; hatched bars, responses to HWA challenge after pretreatment with a combination of L-732138 and SR-48968 (L + SR); cross-hatched bars, responses to HWA challenge after pretreatment with L-732138, the selective NK-1 antagonist (L; 6 mg/kg iv), alone; filled bars, responses to HWA challenge after pretreatment with SR-48968, the selective NK-2 antagonist, alone (SR; 1 mg/kg iv); shaded bars, responses to HWA challenge after pretreatment with formoterol (10 μg/kg iv), the β_2 agonist. See the legend of Fig. 1 for further explanations. Data are means ± SE; five rats in each group. * Significantly different from the response to HWA challenge in the control (vehicle) group ($P < 0.05$).

airways (120.0 ± 29.5 ng/mg; $P > 0.05$, $n = 5$; Fig. 2A), but reduced it by 40% in the lung tissue (18.4 ± 2.0 ng/mg; $P < 0.05$, $n = 5$; Fig. 2B).

3.4. Series 4

Pretreatment with formoterol completely abolished the HWA-induced increase in Evans blue content in Ova-sensitized rats: 15.7 ± 3.2 ng/mg ($P < 0.01$, $n = 5$; Fig. 2A) in major airways, and 5.7 ± 0.5 ng/mg ($P < 0.01$, $n = 5$; Fig. 2B) in lung tissue.

4. Discussion

Results from this study have demonstrated that an increase in T_{tr} to 40.3 °C after hyperventilation of HWA for 2 min evoked a pronounced increase in the Evans blue content in both major airways and lung tissue in Ova-sensitized rats (Fig. 1). In contrast, the same increase in T_{tr} generated by the HWA challenge did not induce any significant increase in Evans blue content in control rats (Fig. 1). Furthermore, the airway extravasation induced by HWA challenge in sensitized rats was completely prevented by pretreatment with a combination of selective NK-1 and NK-2 antagonists, clearly indicating the critical role of endogenously released tachykinins in this effect (Fig. 2). More importantly, our results further demonstrated a primary role of NK-1 receptors in this action of tachykinins since pretreatment with the NK-1 antagonist alone abolished the airway extravasation response to HWA challenge (Fig. 2).

Tachykinins consist of a family of structurally-related small peptides that share a common carboxyl terminal amino acid sequence. In the respiratory tract, two major types of tachykinins have been identified: SP and NKA; they are synthesized mainly in the cell bodies of bronchopulmonary C-fiber afferent nerves, co-localized and co-released form the sensory terminals upon stimulation. SP and NKA have preferential affinities for NK-1 and NK-2 receptors, respectively (Steinhoff et al., 2014). Activation of the NK-1 receptor by SP causes an increase in vascular permeability to plasma macromolecules and adhesion of leukocytes to the vascular endothelium; this effect takes place primarily at the postcapillary venules (Baluk et al., 1998; McDonald, 1988). Activation of the NK-1 receptor also causes smooth muscle relaxation in the arterioles and induces vasodilatation in microvessels. Together, they can cause protein extravasation, mucosal swelling and edema in the airways, which in turn can lead to a reduced airway luminal caliber and an increase in airway resistance.

Several factors are probably involved in the extravasation response observed in this study. One possible mechanism is that chronic airway inflammation may have increased pulmonary C-fiber sensitivity to the HWA challenge in Ova-sensitized rats, which is in agreement with our recent observation in patients with asthma; the same HWA hyperventilation challenge evoked a more intense response of cough and reflex bronchoconstriction in asthmatics than in healthy individuals (Hayes et al., 2012). Indeed, a preliminary study carried out in our lab has shown that the same increase in T_{tr} induced by the HWA challenge evoked a greater intensity of discharge of bronchopulmonatry C-fiber afferents in Ova-sensitized rats (Lin and Lee, 2013), which presumably caused a larger quantity of tachykinins to be released from these sensory endings. In addition, previous investigators have demonstrated that Ova-sensitization increased the amount of tachykinins synthesized and released in guinea pig airways (Fischer et al., 1996). Chronic allergic inflammation also induced a phenotypic switch in the tachykinergic innervation of airways (Myers et al., 2002). Taken together, all these mechanisms may have contributed, to some extent, to the pronounced protein extravasation response to the HWA challenge in the Ova-sensitized airways.

Our results showed that the degree of the HWA-evoked extravasation in Ova-sensitized rats was more pronounced in the trachea and major bronchi than that in the lung tissue (Fig. 1). This difference was probably related to an expected greater increase in temperature generated by the HWA inhalation challenge in the major airways than that in the peripheral airways and distal regions of the lung. We should also point out that the

Evans blue content measured in the lung tissue included that from both lung parenchyma and intrapulmonary airways in this study.

A recent study of our lab has shown that the HWA-induced increase in airway resistance in Ova-sensitized Brown–Norway rats was effectively prevented by a pretreatment with formoterol (Hsu et al., 2013). This observation led us to suggest a possible involvement of airway smooth muscle contraction in the HWA-evoked increase in airway resistance because of the potent dilating effect of formoterol on airway smooth muscles. However, selective β_2 agonists such as formoterol are also known to exert other potent therapeutic effects during acute lung injury, including edema clearance and anti-inflammatory effects (Groshaus et al., 2004). Indeed, previous investigators have demonstrated that activation of β_2 adrenoceptors expressed in the airway vascular endothelial cells increased the intracellular cAMP level in these cells, which resulted in a reduction of the number but not the size of endothelial gaps in postcapillary venules, and diminished the protein extravasation (Baluk and McDonald, 1994). These findings may, therefore, provide the explanation for the effective blocking effect of formoterol on the HWA-evoked airway extravasation in Ova-sensitized rats. Although both formoterol and L-732138 appeared to act on the postcapillary venule endothelial cells, whether their actions are mediated through the same cellular mechanisms is not known since activation of NK-1 receptors can also initiate other G protein-mediated signaling pathways such as activations of phospholipases C_β and A_2, in addition to adenylyl cyclase (Steinhoff et al., 2014).

In summary, an increase in airway temperature within the physiological range induced protein extravasation in major airways and lung tissue in Ova-sensitized rats, but not in control rats. This response was mediated through an activation of the NK-1 receptor by tachykinins released from bronchopulmonary C-fiber nerve endings.

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Hypersensitivity of vagal pulmonary C-fibers induced by increasing airway temperature in ovalbumin-sensitized rats

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Lin YJ, Lin RL, Khosravi M, Lee LY. Hypersensitivity of vagal pulmonary C-fibers induced by increasing airway temperature in ovalbumin-sensitized rats. *Am J Physiol Regul Integr Comp Physiol* 309: R1285–R1291, 2015. First published September 2, 2015; doi:10.1152/ajpregu.00298.2015.—Our recent study has shown that hyperventilation of humidified warm air (HWA) triggered cough and reflex bronchoconstriction in patients with mild asthma. We suggested that a sensitizing effect on bronchopulmonary C-fibers by increasing airway temperature was involved, but direct evidence was lacking. This study was carried out to test the hypothesis that HWA enhances the pulmonary C-fiber sensitivity in Brown-Norway rats sensitized with ovalbumin (Ova). In anesthetized rats, isocapnic hyperventilation of HWA for 3 min rapidly elevated airway temperature to a steady state of 41.7°C. Immediately after the HWA challenge, the baseline fiber activity (FA) of pulmonary C-fibers was markedly elevated in sensitized rats, but not in control rats. Furthermore, the response of pulmonary C-fibers to right atrial injection of capsaicin in sensitized rats was significantly higher than control rats before the HWA challenge, and the response to capsaicin was further amplified after HWA in sensitized rats ($\Delta FA = 4.51 \pm 1.02$ imp/s before, and 9.26 ± 1.74 imp/s after the HWA challenge). A similar pattern of the HWA-induced potentiation of the FA response to phenylbiguanide, another chemical stimulant of C-fibers, was also found in sensitized rats. These results clearly demonstrated that increasing airway temperature significantly elevated both the baseline activity and responses to chemical stimuli of pulmonary C-fibers in Ova-sensitized rats. In conclusion, this study supports the hypothesis that the increased excitability of these afferents may have contributed to the cough and reflex bronchoconstriction evoked by hyperventilation of HWA in patients with asthma.

asthma; airway inflammation; cough; bronchoconstriction; TRPV1

HYPERTHERMIA can occur under normal physiological condition as the results of increased metabolic rate and/or hindered heat dissipation, such as during exercise. Hyperthermia is also found under pathophysiological conditions, for example, in patients who suffer from severe fever or heat stroke. In addition, inflammatory reaction can cause an increase of local tissue temperature. Asthma is an airway inflammatory disease; indeed, a previous study has reported that exhaled breath temperature in average was 2.7°C higher in allergic asthmatic children than in healthy children, and the increase in temperature was closely correlated with the increases in exhaled nitric oxide concentration as well as the number of eosinophils in the induced sputum (39).

In a recent study, we have reported that hyperventilation of humidified warm air (HWA) evoked an immediate and reversible bronchoconstriction (twofold increase in airway resistance) in patients with mild and stable asthma, but not in healthy subjects (15). The HWA-induced bronchoconstriction in these patients was completely prevented by pretreatment with ipratropium, indicating an involvement of cholinergic reflex. Breathing HWA also triggered coughs accompanying the bronchoconstriction in these patients, further suggesting that activation of airway sensory nerves was involved (15). However, direct evidence is still lacking.

Among the sensory nerves innervating the lung and airways, a majority (~75%) are unmyelinated bronchopulmonary C-fibers (20). These sensory afferents exhibit distinct sensitivity to inhaled irritants (e.g., acid aerosol, sulfur dioxide, ammonia, etc.) and endogenous inflammatory mediators (e.g., hydrogen ion, eosinophil granule-derived cationic proteins, and certain metabolites of arachidonic acid, etc.) (8, 16, 22, 26, 27). Activation of these bronchopulmonary C-fiber afferents is known to elicit reflex responses such as bronchoconstriction and cough (8, 26, 27). One of the characteristic features of these afferents is the expression of transient receptor potential vanilloid type 1 receptor (TRPV1) in the nerve endings (16, 46). TRPV1 is a polymodal and nonselective cation channel (7, 38) that can be activated and sensitized by an increase in temperature within the normal physiological range (35, 36).

Furthermore, the overexpression of TRPV1 in bronchopulmonary sensory nerves was demonstrated in Brown-Norway rats actively sensitized by chronic inhalation of ovalbumin (Ova) aerosol (51), an established animal model of allergic asthma (10). In Ova-sensitized rats, acute inhalation challenge of Ova aerosol produced both early- and late-phases of bronchoconstriction, demonstrating the airway hyperreactivity to the antigen challenge. The bronchomotor responses to methacholine challenge were also markedly elevated in Ova-sensitized rats, indicating airway hyperresponsiveness to nonspecific bronchoactive challenge (50). Furthermore, accompanying the airway hyperreactivity, differential cell counts of the bronchoalveolar lavage fluid (BALF) clearly showed the inflammatory cell (eosinophils, neutrophils) infiltration in the airways of sensitized animals. Together, these pathophysiological features induced by Ova sensitization in Brown-Norway rats closely resemble the clinical observations in human allergic asthma, despite certain differences and limitations (10, 14, 42, 45).

On the basis of these findings, we hypothesize that an increase in airway temperature by hyperventilation of HWA elevates the baseline activity and excitability of vagal pulmonary C-fibers in asthmatics. Because a direct recording of bronchopulmonary C-fibers cannot be performed in asthmatic patients, we tested this hypothesis in Ova-sensitized Brown-Norway rats in the present study.

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MATERIALS AND METHODS

The experimental procedures described below were in accordance with the recommendation in *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health and were also approved by the University of Kentucky Institutional Animal Care and Use Committee.

Animal sensitization. The protocol of Ova sensitization used in this study was identical to that reported in details in our recent studies (50, 51). Adult male Brown-Norway rats (age: 3~4 mo) were randomly divided into control and sensitized groups. Sensitized rats received an initial intraperitoneal injection of a solution containing 2 mg Ova in 1 ml Imject Alum as an adjuvant. Three days later, the sensitized rats were exposed to Ova aerosol for 15 min each time, three times a week for 3 wk. During exposure, the conscious rat was placed in a Plexiglas restrainer (University of Kentucky, Center for Manufacturing) and breathed spontaneously and continuously through a nose cone connected to a free stream of air-aerosol mixture under a negative-pressure exhaust hood. Ova solution (wt/vol concentration: 1.25% in isotonic saline) was nebulized and delivered by an ultrasonic nebulizer (model 099HD; DeVilbiss, Somerset, PA) at a droplet size ranging 0.5–5 μm. Control rats received the intraperitoneal injection of Imject Alum alone and aerosol inhalation of the vehicle (isotonic saline) aerosol following the identical procedures.

Animal preparation. One day after the last inhalation exposure to Ova aerosol, rats were initially anesthetized with an intraperitoneal injection of α-chloralose (100 mg/kg) and urethane (500 mg/kg) dissolved in a 2% borax solution; supplemental doses (one-tenth of the initial dose) of the same anesthetics were injected intravenously to maintain abolition of pain reflexes elicited by pinching the tail. One femoral artery was cannulated for recording the arterial blood pressure (ABP) with a pressure transducer (model P23AC; Statham, Hato Rey, Puerto Rico). For administration of pharmacological agents, a catheter was inserted into the left jugular vein and advanced until its tip was positioned just above the right atrium. A short tracheal cannula was inserted just below the larynx via a tracheotomy. Tracheal pressure (P_{tr}) was measured by a transducer (MP45-28; Validyne, Northridge, CA) via a side port of the tracheal cannula. Body temperature was maintained at ~36°C by means of a heating pad placed under the animal lying in supine position.

Electrophysiological recording of pulmonary C-fiber activity. Single-unit fiber activities of vagal pulmonary C-fibers were recorded in anesthetized, closed-chest rats, and the lung was artificially ventilated with a respirator (model 7025; UGO Basile, Comerio-Varese, Italy). Tidal volume (V_T) and frequency were set at 7~8 ml/kg and 60 breaths/min, respectively, to mimic those of unilaterally vagotomized rats (43). The right cervical vagus nerve was separated from right carotid artery. The caudal end of the cut right vagus nerve was placed on a small dissecting platform and immersed in a pool of mineral oil. A thin filament was teased away from the desheathed nerve trunk and placed on a platinum-iridium hook electrode. Action potentials were amplified by a preamplifier (model P511K; Grass Technologies, Warwick, RI) and monitored by an audio monitor (model AM8RS; Grass Technologies). The thin filament was further split until the afferent activity arising from a single unit was electrically isolated. The afferent activity of a single C-fiber was first searched by hyperinflation of the lung (3~4 times V_T) and then identified by the immediate (delay <1 s) response to a bolus injection of capsaicin (Cap, 1.0 μg/kg) into the right atrium. Pulmonary C-fibers typically exhibited a rapid and intense response to Cap injection, but low sensitivity to lung inflation (16); these combined characteristics distinguished them from the high-threshold A-δ pulmonary afferents (49). Finally, at the end of experiment, a midline thoracotomy was made, and the general receptor field of the pulmonary C-fiber was identified by its responses to gentle pressing of the lungs with a blunt-ended glass rod. All physiological signals were recorded on a thermal writer and analyzed by a computer and a data acquisition system (model TS-100; Biocybernetics, Taipei, Taiwan) in 1-s intervals.

HWA challenge. HWA was delivered to the rat's lung using the same preparation that was described in detail previously (17, 29). Briefly, HWA was generated by connecting the outlet of the respirator inspiratory line to an air stone and immersing it in distilled water contained in a bottle that had been placed in a heated water bath. HWA was then delivered directly into the lung via the tracheal tube by the respirator. During HWA challenge, V_T and frequency were set at 14~16 ml/kg and 150 breaths/min, respectively, for 3 min. To prevent arterial hypocapnia and alkalosis, a gas mixture containing 3.5~4.0% CO_2, 21% O_2, and balance N_2 was administered during hyperventilation. We did not measure the end-tidal CO_2 concentration in this study, but our previous studies carried out under identical conditions have shown that both the end-tidal CO_2 and arterial blood pH were maintained unchanged from the baselines during the HWA hyperventilation (17, 29). To continuously measure the temperature in the tracheal lumen (T_{tr}) before, during, and after HWA challenge, we inserted a miniature thermistor (model IT-18; Physitemp, Clifton, NJ, time constant: 0.1 s) to near the distal tip of tracheal tube. T_{tr} was raised to 41~42°C by maintaining the heated water bath temperature at 80°C. The amount of water content in HWA measured in this study was 226 ± 12 mg/l of air.

Experimental protocol and data analysis. In both control and Ova-sensitized rats, the baseline fiber activity (FA) and the response to chemical stimulation were determined before, at 1 min, and 15 min after HWA challenge. Cap, a selective TRPV1 agonist (0.75 μg/kg) and phenylbiguanide [PBG, a selective 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) receptor agonist; 5 μg/kg] were selected as the chemical activators of pulmonary C-fiber afferents. The experiments studying C-fiber responses to Cap and PBG after the HWA challenge were carried out in two separate groups of rats. The volume of each injection was 0.1 ml, which was first injected slowly into the catheter (dead space 0.15 ml) and then flushed into right atrium by an injection of 0.3-ml bolus of saline. FA was continuously recorded and analyzed for 20 s before and 60 s after each injection. The baseline FA was averaged over the 10-s period immediately preceding the injection; and the peak responses were determined by the maximum 2-s and 4-s averages of the FA after the injections of Cap and PBG, respectively. The change in FA (ΔFA) was calculated as the difference between the peak response and baseline FA.

Pharmacological agents. The Ova solution was prepared daily at the concentration described earlier. Stock solutions of Cap (250 μg/ml) were prepared in 10% Tween 80, 10% ethanol, and 80% saline, and that of PBG (1 mg/ml) was prepared in saline. Both solutions were stored at −20°C and prepared daily for injection at the desired concentrations based on the animal's body weight by dilution with isotonic saline. All chemical agents were purchased from Sigma-Aldrich (St. Louis, MO), except Imject Alum (Pierce Biotechnology, Rockford, IL).

Statistical analysis. Data were analyzed with the one-way or two-way repeated-measures ANOVA. For the latter, one factor was the treatment effect of Ova, and the other factor was the effect of HWA challenge. When the ANOVA showed a significant interaction, pair-wise comparisons were made with a post hoc analysis (Fisher's least significant difference). A value of $P < 0.05$ was considered significant. Data are reported as means ± SE.

RESULTS

A total of 32 pulmonary C-fibers were studied in 30 rats in this study; when more than one fiber was recorded in the same animal, the responses were averaged and counted as a single measurement. The average body weight of control rats (299.9 ± 4.8 g, $n = 15$) was significantly higher than the sensitized rats (277.7 ± 6.9 g, $n = 15$; $P < 0.05$) of the same age. Hyperventilation with HWA increased the T_{tr} rapidly from

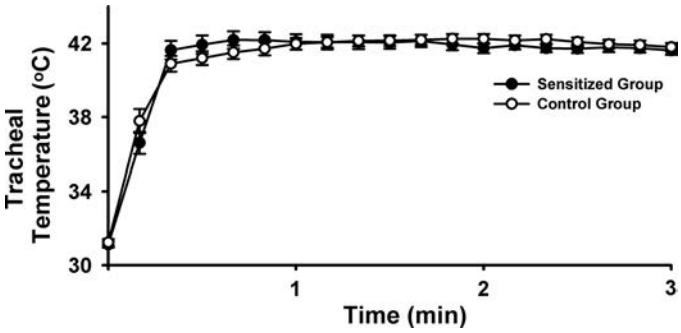

Fig. 1. Change in tracheal temperature during the 3-min hyperventilation with humidified warm air (HWA) in control (open circles) and ovalbumin (Ova)-sensitized Brown-Norway rats (filled circles) that were anesthetized and mechanically ventilated (see text for more details). Data are means ± SE; n = 11 in each group.

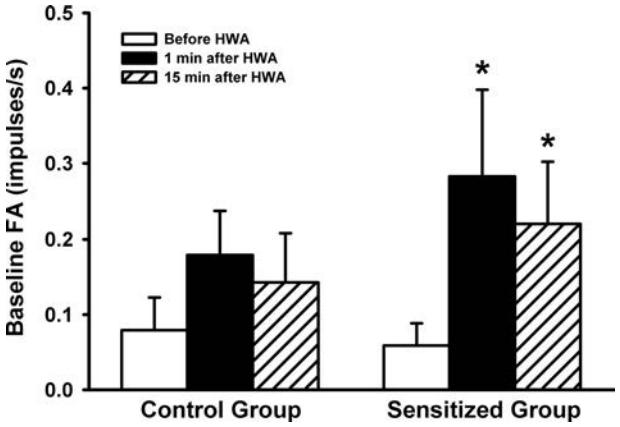

Fig. 3. A comparison of the average baseline fiber activities (FA) before, at 1 min, and 15 min after the HWA challenge between control and sensitized rats. Data were collected from the fibers that are also reported in Figs. 4 and 6. Baseline FA was averaged over 10 s in each fiber. Data are means ± SE; n = 15 in each group. *$P < 0.05$, significantly different from before HWA.

31.2 ± 0.1 to 41.7 ± 0.2°C (n = 22; n = 11 in each group); there was no difference in the peak T_{tr} between control and sensitized groups (Fig. 1). The peak P_{tr} during tidal breathing increased significantly from 5.95 ± 0.20 cmH_2O before HWA to 7.16 ± 0.17 cmH_2O (n = 6; $P < 0.05$) immediately after the HWA challenge in control rats; and from 5.84 ± 0.10 cmH_2O before to 7.74 ± 0.13 cmH_2O (n = 5; $P < 0.05$) after the HWA challenge in Ova-sensitized rats (e.g., Fig. 2, *middle*). These increases in P_{tr} gradually returned toward the baseline after 30 min.

Pulmonary C-fibers showed either no or low and irregular discharge at baseline in both control (baseline FA = 0.08 ± 0.04 imp/s, n = 15) and Ova-sensitized rats (0.06 ± 0.03 imp/s, n = 15) before the HWA challenge (e.g., Figs. 2 and 3). In sensitized rats, the baseline FA increased markedly at 1 and 15 min after the termination of HWA challenge, reaching 0.28 ± 0.12 imp/s (n = 15; $P < 0.05$) and 0.22 ± 0.08 imp/s (n = 15; $P < 0.05$), respectively (Fig. 3). In comparison, the same HWA challenge did not cause any significant increase in the baseline FA in control rats (Fig. 3). The same C-fibers also showed very mild responses to lung inflation (P_{tr} = 30 cmH_2O for 10 s) in both control (0.99 ± 0.45 imp/s, n = 15) and sensitized rats (0.93 ± 0.26 imp/s, n = 15).

Before the HWA challenge, the response of pulmonary C-fibers to Cap was significantly greater in the Ova-sensitized rats (ΔFA = 4.51 ± 1.02 imp/s, n = 8) than in control rats (ΔFA = 1.86 ± 0.27 imp/s, n = 7; $P < 0.05$) (Figs. 2 and 4). At 1 min after the HWA challenge, the C-fiber response to Cap was further amplified in the sensitized rats (ΔFA = 9.26 ± 1.74 imp/s, n = 8; $P < 0.05$), and this potentiation gradually declined and returned to pre-HWA control at 15 min after the HWA challenge (ΔFA = 4.51 ± 1.28 imp/s, n = 8; $P > 0.05$; Figs. 2 and 4). In a sharp contrast, the HWA did not affect the C-fiber response to Cap in control rats (n = 7; $P > 0.05$; Figs. 2 and 4).

Before the HWA challenge, the response of pulmonary C-fibers to PBG was significantly greater in the Ova-sensitized rats (ΔFA = 4.25 ± 1.15 imp/s, n = 7) than in control rats (ΔFA = 0.83 ± 0.22 imp/s, n = 8; $P < 0.05$) (Figs. 5 and 6). At 1 min after the HWA challenge, the C-fiber response to PBG was increased pronouncedly in both control and sensitized rats, but the response was significantly higher in sensitized rats (ΔFA = 8.54 ± 1.70 imp/s, n = 7) than in control rats (ΔFA = 4.10 ± 0.88 imp/s, n = 8; $P < 0.05$) (Figs. 5 and 6). In sensitized rats, this increased response to PBG sustained and remained significantly higher than that in control rats at 15 min after the HWA challenge (Figs. 5 and 6).

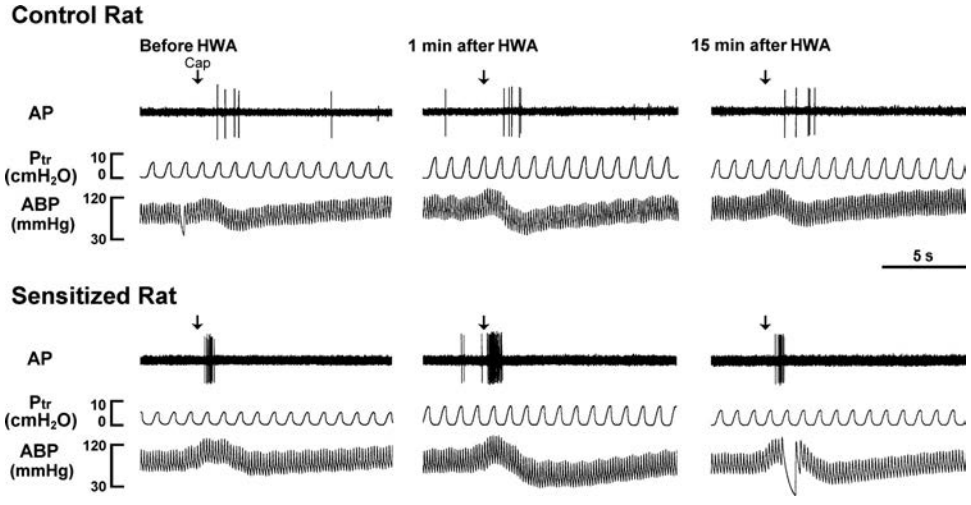

Fig. 2. Experimental records illustrating the effects of the HWA challenge on pulmonary C-fiber responses to capsaicin (Cap, 0.75 μg/kg) in anesthetized, closed-chest, and artificially ventilated rats. Cap solution (0.1 ml) was slowly injected into the catheter (volume 0.15 ml), and then flushed (at arrow) into the right atrium by a bolus of 0.3 ml saline. From *left* to *right*: responses to Cap before, at 1 min, and 15 min after HWA challenge, respectively. AP, action potential; P_{tr}, tracheal pressure, ABP, arterial blood pressure. Receptor location in the control rat (290 g): right upper lobe. Receptor location in the sensitized rat (260 g): right accessory lobe.

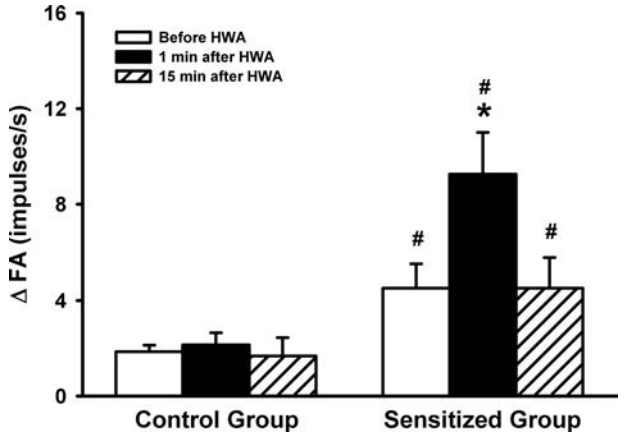

Fig. 4. A comparison of the effects of the HWA challenge on the average peak response of pulmonary C-fibers to right atrial bolus injection of capsaicin (0.75 μg/kg) between control and sensitized rats. ΔFA, difference between the peak response of FA (average over 2-s interval) and the baseline FA (average over 10-s interval) in each fiber. Responses were tested before, at 1 min, and 15 min after the HWA challenge in both control and sensitized rats. Data are means ± SE; $n = 7$ in the control group and $n = 8$ in the sensitized group. *$P < 0.05$, significantly different from before HWA. #$P < 0.05$, significant difference when corresponding data between control and sensitized groups were compared.

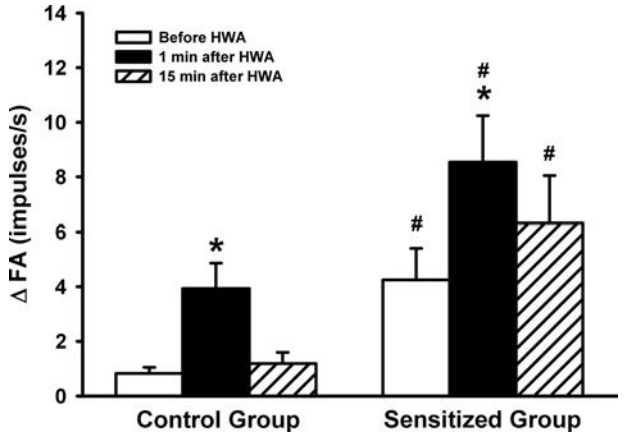

Fig. 6. A comparison of the effects of the HWA challenge on the average peak response of pulmonary C-fibers to right atrial bolus injection of phenylbiguanide (PBG, 5 μg/kg) between control and sensitized rats. ΔFA, difference between the peak response of FA (average over 4-s interval) and the baseline FA (average over 10-s interval) in each fiber. Responses were tested before, at 1 min, and 15 min after the HWA challenge in both control and sensitized rats. Data are means ± SE; $n = 8$ in the control group and $n = 7$ in the sensitized group. *$P < 0.05$, significantly different from before HWA. #$P < 0.05$, significant difference when corresponding data between control and sensitized groups were compared.

DISCUSSION

Results of this study showed that isocapnic hyperventilation of HWA for 3 min markedly elevated the baseline FA of pulmonary C-fibers in Ova-sensitized rats (Fig. 3). Despite the same increase in T_{tr} during the HWA challenge (Fig. 1), there was no significant increase in the baseline FA in control rats (Fig. 3), indicating a heightened stimulatory effect of HWA on pulmonary C-fibers in sensitized rats. Furthermore, the pulmonary C-fiber response to right atrial injection of the same dose of Cap was significantly higher in Ova-sensitized rats than control rats before the HWA challenge, and this enhanced sensitivity to Cap was further amplified after the HWA challenge. A similar pattern of the HWA-induced potentiation in the response to PBG was also observed in sensitized rats. These results clearly demonstrated that increasing airway temperature significantly elevated both the baseline activity and sensitivities to chemical stimuli of pulmonary C-fibers in Ova-sensitized rats. The enhanced C-fiber excitability in Ova-sensitized rats gradually declined and returned to the initial level 15 min after the termination of HWA challenge, suggesting that the effect was not caused by irreversible tissue damage or injury.

In a recent study, we (15) reported that hyperventilation of HWA for 4 min immediately evoked coughs and an increase in airway resistance in patients with mild and stable asthma; at first glance, this finding appeared to be contradictory to the existing knowledge that cold dry air, not warm humid air, triggered bronchoconstriction in asthmatics (3). However, a more in-depth review will reveal that these two seemingly opposite responses are mediated through distinctly different mechanisms. It is known that the primary cause of cold air-induced bronchoconstriction is the injury of airway mucosa, resulting the release of various bronchoconstrictive mediators such as leukotrienes (3). Thus, the airway constriction

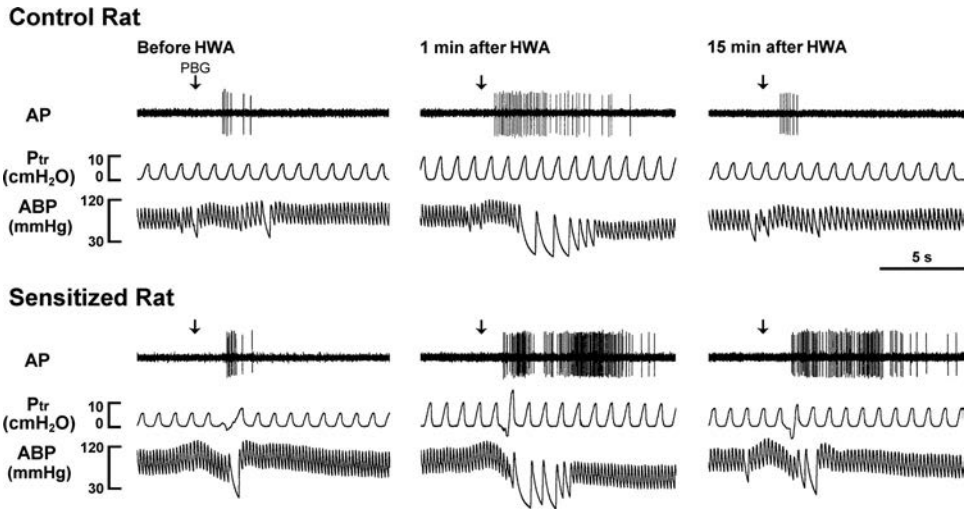

Fig. 5. Experimental records illustrating the effects of the HWA challenge on pulmonary C-fiber responses to phenylbiguanide (PBG, 5 μg/kg). From *left* to *right*: responses to PBG before, at 1 min, and 15 min after HWA challenge, respectively. Receptor location in the control rat (320 g): right upper lobe. Receptor location in the sensitized rat (271 g): right upper lobe. For detailed descriptions of symbols, see Fig. 2.

usually developed slowly, and the response sustained for a much longer duration (1, 3). In contrast, the HWA-induced bronchoconstriction occurred rapidly and was completely prevented by pretreatment with ipratropium, suggesting that it was mediated through cholinergic reflex triggered by activation of airway sensory nerves (15). However, definitive evidence was lacking because a direct recording of bronchopulmonary sensory nerve activity was not feasible in human subjects. Thus the observation in the current study has provided the first direct evidence in support of the hypothesis that an increase in airway temperature by HWA hyperventilation induces both stimulatory and sensitizing effects on the pulmonary C-fibers in allergen-sensitized airways. Indeed, stimulation of these afferents is known to elicit centrally mediated reflex responses, which include bronchoconstriction and mucus hypersecretion via the cholinergic pathway, accompanied by airway irritation and urge to cough (8, 26, 27). Although the mechanisms underlying the sensitizing effect of HWA on pulmonary C-fiber afferents in Ova-sensitized rats are not fully understood, an increase in the expression and/or excitability of TRPV1 is probably a contributing factor (51).

TRPV1 is considered as a biomarker for the C-fiber sensory nerves due to its selective and abundant expression in these neurons. Endogenous TRPV1 activators such as hydrogen ion and certain lipoxygenase metabolites are consistently detected in the BALF, sputum, and/or exhaled breath condensate of patients with airway inflammatory diseases (19, 32). Recent studies further revealed an increase in sensitivity and/or expression of the TRPV1 channel in bronchopulmonary sensory nerves in patients with certain chronic airway diseases (9, 12). The important role of the temperature sensitivity of TRPV1 in regulating airway functions is gaining increasing recognition (11, 24, 38, 40). A previous study carried in our lab has demonstrated that vagal bronchopulmonary sensory neurons isolated in primary culture exhibit distinct thermal sensitivity in whole cell patch-clamp electrophysiological recording experiments (35). Increasing temperature within the normal physiological range evoked inward currents (in voltage-clamp mode), and membrane depolarization and action potentials (in current-clamp mode) in these neurons (35). Furthermore, when the temperature was raised from normal (~36°C) to hyperthermic (~40.6°C) level of the rat body temperature and held constant, the inward current evoked by Cap was significantly increased (36). This potentiating effect was clearly present even at a moderate level of hyperthermia (~39°C). However, it was largely attenuated by selective TRPV1 antagonists capsazepine or AMG 9810 (36) and completely absent in pulmonary nodose/jugular neurons isolated from TRPV1-null mice (37), indicating the potentiating effect of hyperthermia on the TRPV1 chemosensitivity.

The present study demonstrated that this potentiating effect of hyperthermia on the pulmonary C-fiber sensitivity was further enhanced in Ova-sensitized rats. In a previous study, we have reported that chronic airway inflammation induced by Ova sensitization enhanced Cap sensitivity resulting from an increased expression of TRPV1 in these sensory nerves (51). Furthermore, Ova sensitization triggered a phenotypic switch in myelinated bronchopulmonary afferents and upregulated their sensitivity to Cap (50). Neurotrophins such as brain-derived neurotrophic factor and nerve growth factor have been shown to upregulate the expression and sensitivity of TRPV1 in sensory neurons (41, 48); and the synthesis and release of these neurotrophins are known to increase in allergic airways and BALF (4, 31, 47). In addition, other inflammatory mediators released in asthmatic airways (e.g., prostaglandins, protease, etc.) do not activate the TRPV1 directly but can lower its activation threshold (23, 24, 34). Moreover, these inflammatory mediators are also known to cause more generalized hypersensitivity of pulmonary C-fibers to other non-TRPV1 activators (27).

Our results revealed that other non-TRPV1 ion channels were also involved in the HWA-induced sensitizing effect on pulmonary C-fibers in Ova-sensitized rats. The C-fiber response to PBG, a selective agonist of 5-HT_3 receptor, was also significantly augmented in Ova-sensitized rats, and the heightened response was further amplified after the HWA challenge (Figs. 5 and 6). As reported in our previous study, the pulmonary C-fiber response to PBG is not mediated by activation of TRPV1 and cannot be blocked by the TRPV1 antagonists (25). An increase in the tissue temperature can increase the metabolic rate and production of CO_2 and hydrogen ion locally in the airway and lung tissue, which may induce the nonspecific hypersensitivity of pulmonary C-fibers (13). Acute hyperthermia can also elevate the levels of several inflammatory mediators such as arachidonic acid metabolites [e.g., prostaglandin E_2 (PGE_2)] (6) and pro-inflammatory cytokines [e.g., tumor necrosis factor-α (TNF-α)] in the tissue and blood (5); and the sensitizing effects of PGE_2 and TNF-α on bronchopulmonary C-fibers are well documented (18, 23, 30). Furthermore, it has been shown that increasing temperature to ~42°C shifts the TRPV1 channel activation curve from a nonphysiological positive voltage range toward the negative potential in a physiologically relevant voltage range (44). Thus this shift of the voltage-dependent activation curve with a relatively small gating charge may play an important role in the hyperthermia-induced hypersensitivity of these TRPV1-expressing pulmonary sensory neurons.

In conclusion, this study has provided direct evidence in support of the hypothesis that increasing airway temperature elevated the sensitivity of bronchopulmonary C-fibers in the animals sensitized with allergen. This finding explains, at least in part, the observation that breathing hot humid air-triggered coughs and reflex bronchoconstriction in patients with asthma (15) and vigorous cough responses in patients with allergic rhinitis (21). It should be noted that several recent epidemiological and environmental studies have reported a close link of high ambient air temperature to acute asthma exacerbation and airway dysfunction (2, 28, 33). Some of the symptoms reported in those studies, such as cough and dyspnea, are probably related to activation of bronchopulmonary C-fibers (27). However, whether and to what extent the hypersensitivity of bronchopulmonary C-fibers is involved in the airway dysfunction observed in those patients remains to be determined.

Perspectives and Significance

This study has provided the first direct evidence that an increase in airway temperature induces both stimulatory and sensitizing effects on vagal pulmonary C-fibers in an animal model of allergic asthma. This finding offers support to our hypothesis that activation of these sensory nerves is responsible for the cough and reflex bronchoconstriction triggered by

hyperventilation of humidified warm air in patients with mild asthma observed in our recent study (15). Taken together, these studies suggest that exercise-induced hyperventilation in hot humid environment may be a risk factor for asthmatics due to the possibility of triggering dyspnea, cough, bronchospasm, and other symptoms resulting from elevated activity of these sensory nerves.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: Y.-J.L., M.K., and L.-Y.L. conception and design of research; Y.-J.L. and R.-L.L. performed experiments; Y.-J.L., R.-L.L., and L.-Y.L. analyzed data; Y.-J.L., R.-L.L., M.K., and L.-Y.L. interpreted results of experiments; Y.-J.L., R.-L.L., and L.-Y.L. prepared figures; Y.-J.L., R.-L.L., M.K., and L.-Y.L. drafted manuscript; Y.-J.L., R.-L.L., M.K., and L.-Y.L. edited and revised manuscript; Y.-J.L., R.-L.L., M.K., and L.-Y.L. approved final version of manuscript.

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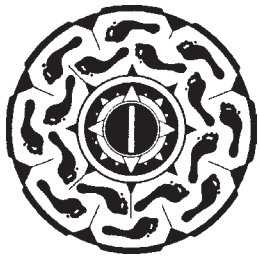


Pulmonary Stresses Induced by Hyperthermia: Role of Airway Sensory Nerves

PI: Lu-Yuan Lee **Org: University of Kentucky**

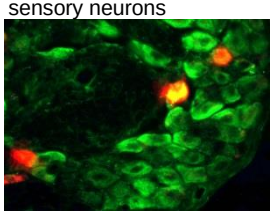
DMRDP

PROBLEM: Under normal physiological condition, activation of pulmonary C-fibers elicits pronounced inhibitory effects on cardiopulmonary function and somatic muscle activity, which plays an important protective role during exertional exercise by limiting muscular performance and thereby preventing over-exertion. However, sustained stimulation of these afferents by hyperthermia may lead to airway dysfunction.

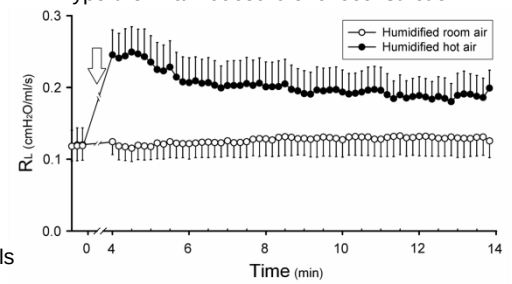
MILITARY RELEVANCE: Soldiers in battle field are often subjected to severe and prolonged stress of hyperthermia. Sustained operations in hot and humid environment can debilitate physiological conditions of soldiers, severely hinder both their physical and mental abilities to perform combat skills and operations, and lead to increased casualties.

HYPOTHESIS: The effect of hyperthermia is primarily mediated through an activation of the TRPV ion channels expressed on vagal pulmonary C-fibers, which leads to airway hypersensitivity. If our hypothesis is correct, soldiers with mild or undetected asthma are likely to be more susceptible to the hyperthermia-induced respiratory/pulmonary stress..

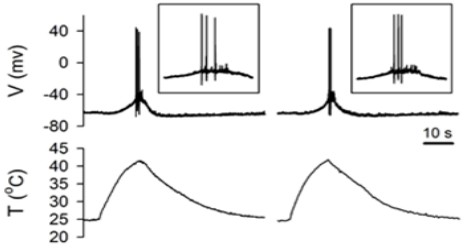
TRPV1-expressing pulmonary sensory neurons



Hyperthermia-induced bronchoconstriction



Hyperthermia-induced action potentials in a rat pulmonary sensory neuron



Human pulmonary function lab

OBJECTIVES: This project will investigate: 1) the acute effect of hyperthermia on airway function and the role of TRPV channels in triggering the bronchospasm; 2) whether this effect is heightened by airway inflammation; and 3) the threshold of thermal stress in generating airway dysfunction in healthy volunteers and mild asthmatics.

EXPERIMENTAL APPROACH: Studies will be performed using three different approaches: in-vivo animal models, isolated pulmonary neurons and human volunteers. Our recently publications and pilot data have clearly demonstrated the feasibility and potential impact of these proposed studies.

SIGNIFICANCE: The results should provide new information for: 1) understanding the mechanism underlying the hyperthermia-induced pulmonary dysfunction; 2) detecting the susceptibility of airway response to heat stress in individual soldiers; 3) developing strategies to safely enhance soldiers' abilities to endure and to perform under heat stress; and 4) establishing safety guidelines to prevent and to reduce injuries caused by hyperthermia during military operations and training.

Timeline and Cost

Activities FY	10	11	12
Enter description of major activity or phase 1; insert or delete rows as needed	Perform animal studies		
Enter description of major activity or phase 2; insert or delete rows as needed		Electrophysiological studies	
Enter description of major activity or phase 3; insert or delete rows as needed		Perform human studies	
Estimated Budget ($K)	220	227	233

Pulmonary Stress Induced by Hyperthermia: Role of Airway Sensory Nerves

(W81XWH-10-2-0189)

PI: Lu-Yuan Lee, Ph.D. Org: University of Kentucky

Award Information

Log Number/Contract Number: W81XWH-10-2-0189
Period of Performance: 30-9-2009 to 29-10-2015
Award Amount: $1,258,852.00
GOR: Brenda K. Bart-Knauer, M.D.

Collaborators:

Don Hayes, M.D.
Mahdi Khosravi, M.D
Deane Roberts, M.D.
Paul B. Collins, B.S., RRT
Richard Kryscio, Ph.D.

Problem Areas

Two no-cost-extension requests were made for this project for the following reasons: 1) Both Dr. Don Hayes and Dr. Deanne Roberts, the co-investigators of this project, left the University of Kentucky unexpectedly, and they had to be replaced. 2) A new group of patients with laryngopharyngeal reflux were proposed, approved and added to the study plan. Consequently, modifications of the IRB and HRPO of our study original protocols had to be approved before we initiated the proposed study.

Key Research Accomplishments

1) Established critical information for documenting the distinct difference in these airway responses to thermal stress between healthy individuals and patients with inflammatory airway diseases.

2) Demonstrated the involvement of TRPV1-sensoty nerves in eliciting these airway response to thermal stress.

3) Obtained novel knowledge and developed new protocol for detecting the susceptibility to heat stress in soldiers with underestimated or overlooked airway hypersensitivity, such as in individuals with mild asthma, allergic rhinitis and laryngopharyngeal reflux.

Next Steps

- To continue the investigations that have been initiated in this project, new grant applications have been prepared and submitted to NIH, and the funding decisions will be made later this year.
- At least one more full manuscript reporting the study that was funded by this project is expected to be submitted for publication in 2016.